MOLECULAR CHARACTERIZATION OF AUTOPHAGY IN METHYLOTROPHIC YEAST *PICHIA PASTORIS*

By

WEIPING YUAN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1998

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. William A. Dunn, Jr., for his excellent guidance and training in my 5-year graduate study. I would also like to extend my gratitude to my committee members, Drs. John P. Aris, Gudrun S. Bennett and Alfred S. Lewin, for their support, help and encouragement of my research. I am in great debt to Dr. Dan Tuttle for his pioneer work in the lab and Dr. Jim Cregg for providing yeast strains, plasmids and the helpful conversations. I would also like to thank the faculty, staff and all other persons at the Department of Anatomy and Cell Biology for the help they rendered and for the stimulating intellectual environment they provided.

My special thanks are reserved for my wife Ying Shen for her wholehearted support in these five years.

TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

LIST OF TABLES vi

LIST OF FIGURES vii

KEY TO ABBREVIATIONS x

ABSTRACT xi

CHAPTERS

1 INTRODUCTION AND REVIEW OF THE LITERATURE 1

The Peroxisome and its Characteristics 2
Peroxisome Biogenesis 2
Peroxisome Functions 3
Methanol Metabolism in *Pichia Pastoris* 5
Autophagy 7
Non-selective Autophagy in Mammalian Cells and Yeast 8
Selective Autophagy in Mammalian Cells and Yeast 11
Genetic Analysis of *Pichia pastoris* 15
Pichia pastoris as a Study Model for Autophagy 15
Classical Genetics 16
Molecular Biology 16
Previous Research of Peroxisome Degradation in Our Lab 19
Chapter Summary 26

2 MATERIALS AND METHODS 27

Yeast, Bacterial Strains and Media 27
Enzyme Assays 29
Isolation of Glucose-Induced Selective Autophagy-Deficient (*gsa*) Mutants 31
Mutant Generation and Isolation 31

Mutant Backcrossing..... 32
Mutant Complementation Analysis..... 33
Molecular Biology..... 34
Yeast Transformation..... 34
Plasmid Isolation and DNA Sequencing..... 35
Northern, Southern and Western Blot Analysis..... 37
Isolation of *PFK1* and *GSA7* Knockouts..... 38
Site-directed Mutagenesis of *PFK1*..... 39
Generation of a HA Epitope Tag in *GSA7* and *YHR171w* gene..... 40
Fluorescence Microscopy and Electron Microscopy..... 41

3 CHARACTERIZATION OF *PICHIA PASTORIS* MUTANTS DEFECTIVE IN GLUCOSE-INDUCED SELECTIVE AUTOPHAGY..... 43

Introduction..... 43
Complementation Group Identification..... 45
Screening for *gsa* Mutants..... 48
Glucose Induced Microautophagy is Defective in *gsa* Mutants..... 49
Morphological Studies of *gsa* Mutants..... 57
Chapter Summary..... 64

4 GSA1 PROTEIN IS PPF1 PROTEIN..... 68

Introduction..... 68
Gsa1-1 Signaling an Upstream Event of Microautophagy..... 68
Microautophagy of Peroxisomes is Defective in *gsa1-1*..... 68
Morphological Study Revealed That a Step Before Peroxisome Sequestration Is Blocked in *gsa1-1*..... 70
pDLT1 Complemented *gsa1-1* Phenotype..... 73
Rescue Study of Different Fragments in the Insert of pDLT1..... 74
Sequencing and Sequence Analysis of pDLT1 Insert..... 75
Northern Blot Analysis of *gsa1-1* Mutant..... 79
Phosphofructokinase Activity Assay in *gsa1*..... 80
Glycolysis Pathway and *gsa* Mutants..... 87
Glycolysis and *gsa* Mutants..... 91
Chapter Summary..... 93

5 PFK1 PROTEIN IS REQUIRED FOR THE INITIATION OF PEROXISOME MICROAUTOPHAGY..... 96

Introduction..... 96

Degradation of Peroxisomes by Microautophagy Requires *PFK1*..... 97
PFK1 Gene Disruption and the Verification..... 97
The *PFK1* Knockout Has the Same Phenotype as *gsa1-1*.... 97
Verification by Site Directed Mutagenesis of *P. pastoris PFK1* Gene of the Distinction between Rescue Function and PFKp Activity..... 100
Normal Pfk1p and Catalytically-inactive pfk1p Complement *Δpfk1*..... 100
Restoration of the Degradation Ability of *Δpfk1 by PFK1*.....104
Morphological Characterization of *Δpfk1*..... 106
Chapter Summary..... 109

6 CHARACTERIZATION OF *GSA7* MUTANTS..... 111

Introduction..... 111
Morphological Studies of *gsa7*..... 111
Recovery of *GSA7* Gene and its Verification..... 112
pYWP7-4 Rescues *gsa7* phenotype..... 112
Identification of *GSA7*..... 114
Sequence Analysis of *GSA7*..... 116
Δgsa7 Generation and its Phenotype Studies..... 119
A HA Tagged Gsa7p Rescues *P. pastoris*..... 120
Chapter Summary..... 122

7 CONCLUSIONS AND PROSPECTS..... 127

Introduction..... 127
Characterization of *gsa* Mutants..... 131
Regulation of the Signaling of Microautophagy by Gsa1p..... 132
Regulation of a Homotypic Vacuolar Membrane Fusion Event of Microautophagy by Gsa7p..... 135
Prospects and Conclusion..... 137

REFERENCES..... 141

BIOGRAPHICAL SKETCH..... 151

LIST OF TABLES

Tables		Page
2-1	Parental and mutant strains of *Pichia pastoris*	28
3-1	Biochemical profiles of *gsa* mutants	53

LIST OF FIGURES

Figure		Page
1-1	Compartmentalization of the pathways involved in methanol metabolism in methylotrophic yeast	6
1-2	Loss of peroxisomal and cytosolic enzymes during ethanol and glucose adaptation	20
1-3	Morphology characterization of peroxisomes during glucose and ethanol adaptation	21
1-4	Model of glucose-induced microautophagy pathway	24
2-1	Maps of plasmids pYM8 and pYM4	36
3-1	A flow chart of mutants characterization	44
3-2	Identification of *gsa* mutants complementation groups	47
3-3	AOX and FDH activities of *gsa* mutants under glucose adaptation	51
3-4	AOX and FDH activities of *gsa* mutants under glucose adaptation	52
3-5	AOX degradation and histidine addition during glucose adaptation	56
3-6	Ultrastructural studies of *gsa1-1*, *gsa2*, *gsa3*, *gsa6*, *gsa7* and *gsa8* mutants under glucose adaptation	62
3-7	Ultrastructural studies of *gsa4* and *gsa5* mutants under glucose adaptation	63
3-8	Microautophagy of peroxisomes and *gsa* mutants in *P. pastoris*	65
4-1	Glucose and ethanol adaptation in parental GS115 and WDY2 (*gsa1-1*)	69

4-2 Morphology of *gsa1-1* and *gsa2* under glucose adaptation at 0h and 3h time points 72

4-3 pDLT1 rescues *gsa1-1* phenotype 76

4-4 Identification of genomic DNA that rescues *gsa1-1* during glucose adaptation 77

4-5 Northern blot analyses of GS115, WDY1 *(gsa1)* and WDY2 *(gsa1-1)* 81

4-6 PFK activity is greatly reduced in WDY2 (*gsa1-1)* cells 82

4-7 Nucleotide and predicted amino acid sequence of *P. pastoris PFK1* gene 85

4-8 Comparison of *PFK1* genes 86

4-9 Glycolysis pathway and the entry point of carbon metabolites 89

4-10 Metabolites in the glycolysis pathway and their induction of the degradation of AOX 90

4-11 Glycolysis enzyme activities and *gsa* mutants 92

5-1 Generation of the *PFK1* knockout 98

5-2 Glucose-induced degradation of AOX and FDH in PPF1 and WDKO1 ..99

5-3 Partial sequences comparison of Pfk1p in *P. pastoris* (PP), *S. cerevisiae* (SC) and *Human sapiens* (HU) 100

5-4 Site-directed mutagenesis of *P. pastoris PFK1* gene 102

5-5 Morphology of GS115, *gsa1-1*, and *Δpfk1* during glucose adaptation 107

5-6 Morphology of WDKO1 (*Δpfk1)* and its transformants during glucose adaptation 108

6-1 Morphology of *gsa3* and *gsa7* mutants during glucose adaptation 113

6-2 Verification of *GSA7* 115

6-3 Complete nucleotide and amino acid sequence of *GSA7* 117

6-4 A protein sequence comparison of *GSA7* in *P. pastoris*, *S. cerevisiae*, *S. pombe* and *H. sapiens*..... 118

6-5 *Δgsa7* generation and its verification..... 121

6-6 Detection of a HA epitope tagged Gsa7p and Yhr171wp in transformed *gsa7* 123

7-1 Pathways of peroxisome induction and degradation under glucose and ethanol adaptation in methylotrophic yeast *Pichia pastoris*..... 129

7-2 Microautophagy and macroautophagy of peroxisomes in *P. pastoris*..... 130

7-3 Putative substrate binding and regulatory sites in Pfk1p..... 134

7-4 Pathway of glucose induced peroxisome microautophagy and its relationship with *gsa* mutants..... 139

KEY TO ABBREVIATIONS

AOX: alcohol oxidase
BSA: bovine serum albumin
CCO: cytochrome c oxidase
DSM: diploid selection medium
EAM: ethanol adaptation medium
F1β: mitochondrial F1 ATPase, β subunit
FBP: fructose-1,6- bisphosphatase
FDH: formate dehydrogenase
GAM: glucose adaptation medium
gsa: glucose-induced selective autophagy deficient mutant
Gsap: Gsa protein
GSA: *GSA* gene
Δgsa1: *gsa1* knockout strain
Δgsa7: *gsa7* knockout strain
mRNA: message RNA
MIM: methanol induction medium
ORF: open reading frame
PFK1: phosphofructokinase 1 gene
Pfk1p: phosphofructokinase 1 protein
pfk1: mutated Pfk1 protein
pfk1: mutated *PFK1* gene
SM: sporulation medium
TM: transformation medium
YND: yeast nitrogen base medium with glucose
YNM: yeast nitrogen base medium with methanol
YPD: glucose-containing complete medium

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MOLECULAR CHARACTERIZATION OF AUTOPHAGY IN METHYLOTROPHIC YEASTS *PICHIA PASTORIS*

By

Weiping Yuan

December 1998

Chairman: William A. Dunn, Jr.
Major Department: Anatomy and Cell Biology

Eukaryotic cells adapt to environmental changes by synthesizing and degrading cellular proteins and organelles. Although much is known regarding the biogenesis of cell organelles, the mechanism of turnover of many organelles and proteins through autophagy remains unclear. Our lab has utilized the yeast *P. pastoris* as a study model for the autophagy of peroxisomes during metabolic adaptation. Peroxisomes are induced when *P. pastoris* is grown on methanol. During the adaptation to glucose, peroxisomes are degraded within the yeast vacuole by microautophagy. This process includes glucose signaling, vacuolar recognition and sequestration of peroxisomes, homotypic vacuolar membrane fusion and finally vacuolar degradation of peroxisomes.

In order to better understand the microautophagy process, glucose-induced selective autophagy-deficient (*gsa*) mutants were generated and isolated by their inability to degrade peroxisomal alcohol oxidase (AOX) during glucose adaptation. Eight *gsa* complementation groups (*gsa1 to gsa8*) have been identified and they represent different gene products that control microautophagy processes. These *gsa* mutants fall into four major groups. *Gsa1* belongs to microautophagy initiation mutants. *Gsa4* and *gsa8* are possible autophagy recognition mutants. *Gsa2*, *gsa3, gsa6,* and *gsa7* are possible homotypic vacuolar membrane fusion mutants. *Gsa5* is also a homotypic fusion mutant but its macroautophagy pathway has been turned on during the methanol induction.

Gsa1-1 was transformed with a *Pichia pastoris* genomic DNA library. The gene that complemented the *gsa1-1* phenotype was identified as *phosphofructokinase 1 (PFK1)*. Cellular levels of both *PFK1* mRNA and PFKp activity were greatly reduced in *gsa1-1*. The inability of Δ*pfk1* to degrade AOX could be rescued by either normal *PFK1* or mutant *pfk1* whose catalytic site had been inactivated by a single amino acid mutation. It suggests that the degradation of peroxisomes does not require a catalytically active PFKp. Pfk1p might regulate microautophagy independent of its PFK activity.

WDY7 (*gsa7*) was the second mutant I chose for further genetic study. I recovered a plasmid pYWP7-4 that complemented *gsa7*. The gene was identified as *GSA7*. A knockout strain Δ*gsa7* failed to degrade AOX confirmed

GSA7 is essential for the microautophagy. Gsa7p might participate in a ubiquitin-like pathway to regulate the vacuole membrane fusion.

The study of microautophagy in *Pichia pastoris* provides us a unique opportunity to look into the mechanisms of protein degradation via autophagy. The study I have done and the tools that have become available in recent years will be useful to further elucidate the molecular mechanism of microautophagy in eukaryotic cells.

CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

Some yeast species such as *Candida boidinii, Hansenula polymorpha*, and *Pichia pastoris* are able to utilize methanol as the sole carbon and energy source for their growth and thus are called methylotrophic yeasts. These methylotrophic yeasts metabolize the methanol by enzymes in the peroxisomes and cytoplasm whose synthesis is induced under methanol-growth conditions (Hill et al., 1985). These enzymes as well as the whole peroxisomes are rapidly degraded upon a carbon source switch to such as glucose or ethanol. The study of the biogenesis and degradation processes of peroxisomes under different growth conditions in *Pichia pastoris* and other yeast species has gained great interest in recent years because in humans, an inability to import proteins into the peroxisomes has serious health consequences (Moser and Moser, 1996). Genetic screens for peroxisomal assembly (*pas*) (Gould et al., 1992), deficiency (*per*) (van der Klei et al., 1991), and degradation (*gsa*, *ppd*) (Tuttle and Dunn, 1995, Titorenko et al., 1995) mutants in yeast have been successfully utilized to identify many genes required for these events. Moreover, several genes cloned in these mutants have been found to have human homologous counterparts (Dodt and Gould, 1996). These findings emphasize the usefulness of the yeast

mutant study model and the significance of these studies. In the following sections, I will discuss the existing knowledge relate to peroxisome biogenesis, degradation and autophagy. I will also discuss the methods available to study the mechanism of autophagy in yeast peroxisomes and the previous work that has been done in our lab.

The Peroxisome and its Characteristics

Peroxisome Biogenesis

Peroxisomes are ubiquitous, single-membrane-bound organelles that are involved in many important cellular activities. They were first discovered thirty years ago in a centrifugation fraction of cell components (de Duve, 1965). The peroxisomes are thought to arise by budding or fission from preexisting peroxisomes although evidence for *de novo* synthesis has been presented. Peroxisomes contain neither DNA nor ribosomes. Their proteins are encoded by nuclear genes and synthesized in cytoplasm on free polyribosomes and then post-translationally imported into the preexisting organellar matrix or surrounding membrane (Liu et al., 1995). Almost all peroxisomal proteins are synthesized at their final size. Two peroxisomal targeting sequences (PTS) responsible for correct delivery of matrix proteins to the organelles have been identified. The majority of peroxisomal matrix proteins contain PTS1, a C-terminal tripeptide of the sequence SKL-COOH (Keller et al., 1991). PTS2, with the consensus

sequence RLX5H/QL is located at N-terminal of peroxisomal proteins (Swinkels et al., 1991). Many studies focused on the biogenesis of peroxisomes and the defects in peroxisome assembly and their protein import. In *P. pastoris*, 15 complementation groups of peroxisome assembly mutants (*PAS* mutant) have been identified (Subramani, 1993). Some of the *PAS* genes have been sequenced. *PAS4* gene was identified as a ubiquitin-conjugating enzyme required for peroxisome assembly (Crane et al., 1994). It is an interesting finding since the ubiquitin mediated protein degradation pathway is responsible for protein turnover. We do not yet know if *PAS4* has a role in the peroxisome protein degradation pathway through targeted degradation by the proteasome. McCollum et al. (1993) reported cloning the *PAS8* gene and demonstrated that it is a PTS1 import factor. Subsequent studies showed that it is a PTS1 import receptor and the tetratricopeptide repeat domain of PAS8p is identified as the PTS1 binding region (Terlecky et al., 1995). This finding linked peroxisome protein import and targeting and the peroxisome assembly pathway in the peroxisome biogenesis process.

Peroxisome Functions

Two prominent functions of peroxisomes are the β-oxidation of fatty acids and the oxidation of substrates by different H_2O_2-generating oxidases (Subramani, 1993). Other functions of peroxisomes include lipid biosynthesis, cholesterol biosynthesis and peroxisomal purine metabolism. Peroxisomes are unique in their functional diversity when compared with other cell organelles

such as mitochondria, the nucleus, and organelles of the secretory pathway. Their functions are dispensable in certain nutritional environments and the specific metabolic pathways found in the organelle vary depending upon the organism, the tissue and its environment. This was verified by observations that mutants defective in peroxisome assembly in yeast and human fibroblast cell lines were still viable (Subramani, 1993). Nevertheless, the peroxisome is essential for normal human development, since defects in peroxisome assembly have been identified as the cause of the peroxisome biogenesis diseases.

In methylotrophic yeast *P. pastoris*, when cells were grown on nutrition rich YPD media, yeast maintained a low number of peroxisomes per cell. The size of the peroxisomes is also small. However, peroxisomes can also be made to proliferate or degrade in response to nutritional cues such as methanol or oleic acid (Liu et al., 1992, Subramani, 1993). The process of peroxisome degradation is not as well understood as biogenesis. Cells respond to nutritional clues to reduce the number of peroxisomes via degradation. Such events are exemplified by supplying methanol as growth carbon source to induce peroxisome production and then abolishing the induction by a carbon source switch such as glucose or ethanol. The whole process usually takes less than eight hours to finish (Tuttle and Dunn, 1995). This degradation event provides us a unique opportunity to investigate the mechanisms of peroxisome degradation.

Methanol Metabolism in *Pichia pastoris*

When methanol is added to the *P. pastoris* growth media as the only carbon source, methanol readily enters the cell and peroxisome for metabolism. The peroxisomal enzyme alcohol oxidase (AOX) is the first enzyme in the methanol assimilation pathway. AOX is a homooctameric protein of ~ 74 kD, each subunit containing a flavin adenine dinucleotide molecule (FAD) as a prosthetic group (van der Klei et al., 1991). It uses O_2 as the electron acceptor, oxidizes methanol and produces hydrogen peroxide and formaldehyde (Gleeson and Sudbery, 1988). The hydrogen peroxide is reduced by peroxisomal catalase to H_2O and O_2. Formaldehyde exits the peroxisome and is catalyzed in cytosol by formaldehyde dehydrogenase and formate dehydrogenase (FDH) to CO_2 providing an energy source in the form of $NADH_2$ (Tolbert, 1981). Formaldehyde enters the xylulose 5-phosphate cycle to provide a carbon source for amino acid synthesis (Fig. 1-1).

One characteristic of AOX is that it is not detectable in glucose-grown cells but inducible under methanol growth condition. Another is that it has a low affinity for its substrate and thus large quantities of the enzyme (up to 30% of the total cellular soluble proteins) are needed for yeast to grow on methanol as the sole carbon source. When the carbon source for growth is switched from methanol to glucose or ethanol (glucose or ethanol adaptation), yeast peroxisomes and thereby also AOX are degraded rapidly in its vacuole via autophagy (Tuttle et al., 1993). The inactivation of AOX activity is paralleled by

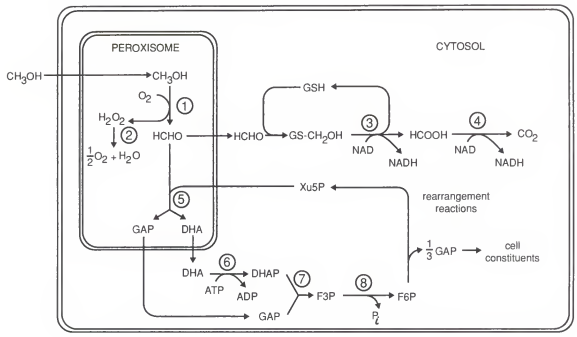


Figure 1-1. Compartmentalization of the pathways involved in methanol metabolism in methylotrophic yeast. Methanol is metabolized first in peroxisome and then in the cytosol for further degradation. Its metabolism pathway is shown in the figure above and the enzyme names are listed below. The pathway is responsible for the generation of GAP for the production of biomass and the $NADH_2$ for energy generation. 1 = Alcohol oxidase; 2 = catalase; 3 = formaldehyde dehydrogenase; 4 = formate dehydrogenase; 5 = dihydroxyacetone synthase; 6 = dihydroxyacetone kinase; 7 = fructose 1,6-bisphosphate aldolase; 8 = fructose 1,6-bisphosphate phosphatase (Adapted from van der Klei et al., 1991)

its degradation of peroxisomes in this process (Veenhuis et al., 1983) and the disappearance of AOX activity correlates with the degradation of AOX protein and autophagic loss of peroxisomes. The degradation of peroxisome is proteinase A and B dependent (Tuttle and Dunn, 1995). These characteristics of AOX provide us an excellent biochemical index for studying peroxisome degradation under different nutritional adaptation conditions. It also provides us an easy enzyme assay to screen autophagy mutants.

Autophagy

Cells respond to changes in the environment by eliminating cell components that are no longer needed. The amino acids then are released and used as an energy source or for the synthesis of new proteins. This provides the organism with added adaptability that outweighs the metabolic costs such as ATP used in protein degradation pathways (Olson et al., 1992). The continual turnover of cellular proteins also prevents the accumulation of a variety of deleterious nonenzymatic modifications such as oxidation, deamination, glycosylation of the abnormal proteins that are deleterious to the cells (Klionsky, 1997). The degradation of protein in the cell is mainly via lysosomal proteolytic pathways, proteolytic pathways of non-lysosomal organelles and cytosolic proteolytic pathways. In the following section, I will mainly discuss the existing knowledge of autophagy pathways in mammalian cells and yeast cells.

Non-Selective Autophagy in Mammalian Cells and Yeast

Autophagy means self-eating. It is used to refer to the process whereby cytoplasmic constituents are degraded in a vacuole-dependent manner. It has been recognized as a highly regulated non-selective and sometimes selective process for the degradation of cellular proteins and organelles in eukaryotic cells. Mammalian cells respond to amino acid starvation and other environmental changes (e.g., heat shock) by activating the degradation of proteins via non-selective autophagy (Lardeux and Mortimore, 1987, Mortimore et al., 1989, Kopitz et al., 1990, Dunn et al., 1994). Both microautophagy and macroautophagy have been observed in mammalian cells. Microautophagy is the process whereby cellular components are sequestered directly into the lysosome. Regions of the cytoplasm are surrounded by invagination of the lysosomal membrane or by finger-like protrusions of the lysosome. Upon fusion of the lysosomal membrane, intralysosomal vesicles are formed containing the sequestered cellular components (Ahlberg and Glaumann, 1985, Mortimore et al., 1989). It is believed that multivesicular bodies arise from such autophagic events. Macroautophagy is the process whereby cellular components are sequestered first within an autophagosome (Dunn, 1990a). The autophagosome originates from the rough endoplasmic reticulum in mammalian cells and, then fuses with a lysosome. Once within the lysosome, the proteins are degraded to their monomeric subunits by the concerted action of exopeptidases and endopeptidases (Dunn, 1990a, 1990b).

Many strains of yeast are capable of activating autophagy in response to nutritional changes (Veenhuis et al., 1983, Tuttle et al., 1993, Baba et al., 1994, Tuttle and Dunn, 1995, Chiang et al., 1996). The main organelle for autophagy in yeast is its vacuole, the equivalent of lysosome in mammalian cells. In order to better define the molecular events of autophagy, mutants defective in autophagy have been used to study the mechanism of autophagy in yeast *S. cerevisiae, P. pastoris and H. polymorpha*. During nitrogen starvation, cells of a normal strain of *S. cerevisiae* respond to this stress by degrading cellular contents non-specifically via autophagy. Ohsumi's group found in a *S. cerevisiae* mutant lacking proteinase B, "autophagic bodies" containing cytosolic contents were accumulated in the vacuoles during nitrogen starvation. (Takeshige et al., 1992). They (Tsukada and Ohsumi, 1993) used this strain to generated *apg* (autophagy) mutants which failed to accumulate autophagic bodies within the yeast vacuole. Strains with *apg* mutation failed to deliver celluar contents to the yeast vacuole and are thus the non-selective autophagy mutants. Their ultrastructural data suggested that the autophagic process in yeast is essentially similar to that of the lysosomal system in mammalian cells (Baba et al., 1994). The *apg* mutants fell into 15 complementation groups. The characterization of these mutants is underway and several novel genes have been identified, sequenced and their functions in the process of autophagy are being studied (Shirahama et al., 1997; Matsuura et al., 1997; Noda and Ohsumi, 1998).

The *aut* (autophagy) mutants identified by a colony screening procedure by Thumm et al. (1994) are defective in the degradation of cytosolic fatty acid synthase during carbon starvation. This served as a selection marker for rapid isolation of autophagocytosis yeast mutants defective in vacuolar breakdown of fatty acid synthase during starvation. Further identification and characterization of the genes of *apg* and *aut* mutants will reveal the mechanisms and function of autophagocytosis. It will also reveal why all autophagy mutants have defects in their sporulation process. It appears that some of these autophagy mutations are allelic (Harding et al., 1996, Scott et al., 1996). However, it is not known whether or not these mutations affect macroautophagy induced by re-administering glucose in *S. cerevisiae*. It will be interesting to see if there is an overlap in the degradation pathway in non-selective and selective autophagy pathways and the diverging point of these two pathways.

Several labs including ours have observed similar autophagy processes in methylotrophic yeast during the protein and cell organelle degradation process. Veenhuis et al (1978, 1981) observed in methanol-induced *H. polymorpha* cells, when the carbon source was switched to ethanol, the earliest change was the appearance of a variable number (2 to 12) layers of electron-dense membranes surrounding peroxisomes. These membranes surrounded individual peroxisomes within a cluster and appeared to sequester a given peroxisome into an autophagosome and then deliver its contents to the yeast vacuole. The process is analogous to macroautophagy in mammalian cells.

Tuttle and Dunn (1995) found that when growth carbon source was switched from methanol to ethanol, the process of peroxisome autophagy in *P. pastoris* includes an intermediate stage in which individual peroxisomes were sequestered into autophagosomes by wrapping membranes and the autophagosomes then fused with the vacuole, a process similar to macroautophagy. In the glucose-induced peroxisome degradation pathway, he observed that autophagy began with the engulfment of clusters of peroxisomes by finger-like protrusions of the vacuole and the contents were degraded, a process analogous to microautophagy in mammalian cells.

Selective Autophagy in Mammalian Cells and Yeast

Although non-selective autophagy is considered to be responsible for the bulk turnover of proteins that occurs in response to nutritional starvation or developmental stress (Takeshige et al., 1992), several studies suggested that selective autophagy also plays an important role in regulation of protein and organelle levels in mammalian and yeast cells. Zellweger syndrome is a well-known prototypic peroxisomal disorder in the newborn (Lazarow and Fujiki, 1985). Normal peroxisomes appeared to be absent in cells of these patients and peroxisomal enzymes remained in the cytosol due to impaired assembly of peroxisomes (Shimozawa et al., 1992). However, the fibroblasts from these patients were propagated readily in tissue culture. Meijer's group found that in Zellweger fibroblasts, most peroxisomal ghosts in fact contained lysosomal hydrolases (Heikoop et al., 1992). The treatment with the autophagy inhibitor 3-

methyladenine caused an increase in the number of peroxisome ghosts though peroxisomal functions were impaired. It suggested that peroxisomal ghosts of Zellweger cells were selectively degraded by autophagy. In another study (Luiken et al., 1992) in, they found that peroxisomal enzymes fatty acyl-CoA oxidase and catalase were preferentially degraded in the isolated hepatocytes of clofibrate-fed rats. This increased degradation of the peroxisomal enzymes was prevented by 3-methyladenine, an inhibitor of macroautophagic sequestration. Long-chain fatty acid could inhibit this degradation too. They concluded that preferential autophagy of peroxisomes exists.

In yeasts, independent studies showed evidence that selective autophagy plays an important role to adjust their cellular composition according to environment changes. Veenhuis and coworkers studied selective inactivation of AOX in two peroxisome-deficient (*per*) mutants in *Hansenula* (Veenhuis et al., 1983, van der Klei et al., 1991, Titorenko et al., 1995). They found that these two *per* mutants synthesized AOX but most of AOX remained in cytosol. Only a small percentage of AOX was degraded under the glucose adaptation condition. These data indicated that degradative inactivation of AOX in *H. polymorpha* is strictly dependent on the localization of the enzyme inside peroxisomes and the mechanism triggering this process is not directed against AOX protein, but instead, to the membrane surrounding the organelle. In another study, they also observed that not all peroxisomes were degraded in *H. polymorpha* during the carbon source switch (Veenhuis et al., 1983). One or few small peroxisomes

escaped degradation and subsequently served as the target organelle for newly synthesized matrix proteins in the new growth environment. In their recent study (Titorenko et al., 1995), they have generated mutants impaired in the selective degradation of peroxisomes in *H. polymorpha* (peroxisome degradation-deficient, *pdd*). Of the seven mutants they studied, two complementation groups have been identified with two possible responsible genes *PDD1* and *PDD2*. The function of their products in selective autophagy of AOX-containing peroxisomes is under study. The PDD1p is involved in the initial signaling events for sequestration of the organelle since no multilayer membrane sequestration of peroxisomes was observed in *pdd1* when compared with wild type cells under ETOH adaptation. In *pdd2* mutants, sequestration did occur since the multilayer membrane appeared and wrapped peroxisomes but subsequent fusion of vacuole membrane did not occur. Immunocytochemical detection of alcohol oxidase protein in the vacuole was not observed in any cases. They concluded that PDD2p is essential for mediating the second step in selective peroxisome degradation, namely, fusion and subsequent uptake of sequestered organelles into the vacuole. The model they established for the study of peroxisome maintenance and proliferation, sorting, folding, assembly of matrix proteins, synthesis and function of the peroxisome membrane, and peroxisome turnover in *H. polymorpha* (van der Klei and Veenhuis, 1996) is similar to what we have established in *P. pastoris* (Tuttle and Dunn, 1995).

In *S. cerevisiae*, carbon and nitrogen starvation stimulates non-selective macroautophagy (Takeshige et al., 1992, Egner et al., 1993, Baba et al., 1994). However, data for selective degradation by microautophagy have been reported when yeast cells was switched from oleic acid medium to the one containing glucose (Chiang and Schekman, 1991). When cells were replenished with glucose, a key gluconeogenic enzyme, fructose-1,6-bisphosphatase (FBPase) was selectively targeted from the cytosol to the yeast lysosome (vacuole) for degradation (Chiang et al., 1996). Peroxisomes have been identified as the target organelles to be delivered to the vacuole for degradation when cells were replenished with glucose. They have generated vacuolar import and degradation-deficient (*vid*) mutants and these mutants were placed into 20 complementation groups. FBPase degradation was blocked in all these mutants (Hoffman and Chiang, 1996). They identified a novel type of vesicles in the cytosol specific to vacuolar protein degradation pathway. These vesicles are intermediate in the FBPase degradation pathway which is different to all established vesicles (Huang and Chiang, 1997). One novel responsible gene *VID24* has been recovered (Chiang, 1997) Vip24p is a 41kD protein and is induced by glucose addition and localized to the intermediate vesicles as a peripheral protein. In the absence of Vid24p, FBPase accumulates in the intermediate vesicles. It seems that this protein plays a critical role to deliver FBPase from the intermediate vesicles for vacuolar degradation. The study clearly showed that the selective protein degradation is present in *S. cerevisiae*.

Our lab was the first to study selective autophagy in *P. pastoris* using microautophagy mutants. As stated in the beginning of this chapter, we have identified two distinct peroxisome degradation pathways namely, microautophagy and macroautophagy. We have shown that the autophagy of peroxisome needs synthesis of new molecules.

Genetic Analysis of *Pichia pastoris*

Pichia pastoris as a Study Model for Autophagy

Yeast is a much simpler system to study autophagy processes than mammalian cells systems. Yeast cells are easy to grow and handle. Their biochemical and morphological characterization can be done quickly. They are one cell eukaryotic organisms and have a much smaller genome and thus fewer genes involved in the autophagy process. When comparing the cells of *P. pastoris* with *S. cerevisiae* in the peroxisome degradation process, under glucose induction, peroxisomes are larger, fewer in number, and much easier for biochemical and morphological studies in *P. pastoris* than *S. cerevisiae*. The peroxisome degradation process is also much faster in *P. pastoris*. Eight hour glucose adaptation results in complete peroxisome degradation while in *S. cerevisiae*, the degradation of peroxisomes under glucose induction needs at least 24 hrs. The measurement of degradation is difficult in *S. cerevisiae* because the growth of *S. cerevisiae* under glucose induction results in a dilution

of peroxisomes per cell in a 24 hour period (Chiang et al., 1996; Tuttle, unpublished data).

Classical Genetics

Pichia pastoris has many advantages in genetic studies. It is ascosporogenic and exists in one of two mating types. The life cycle of *Pichia pastoris* is characterized by defined haploid and diploid stages (Gleeson and Sudbery, 1988, Cregg et al., 1990) and it can be maintained indefinitely as vegetative haploids. However, upon nutritional limitation, particularly for nitrogen, mating occurs and diploid cells are formed. The mating type can switch between two opposite strains under poor nutritional conditions at high frequency. These key features allow the isolation and phenotypic characterization of mutants in the chemically mutagenized *P. pastoris*. It also allows us to characterize the phenotypic identification and the complementation groups of mutants (Cregg et al., 1990). Dr. Cregg's group (Liu et al., 1992) developed an efficient screening method for peroxisome-deficient mutants in *P. pastoris*. The screen relies on the unusual ability of *P. pastoris* to grow on two carbon sources, methanol and oleic acid, both of which absolutely require peroxisomes to be degraded. A collection of 280 methanol utilization-defective (*mut*) mutants were isolated, and organized into 46 complementation groups.

Molecular Biology

P. pastoris has also been developed to be an efficient transformation system for the introduction of replicating plasmids by using *E. coli--P. pastoris*

shuttle vectors. The system is based on a histidinol dehydrogenase-defective mutant host of *P. pastoris* and a modified version of the spheroplast fusion-gene transfer procedure (Cregg et al., 1985). The *His4* gene of *P. pastoris* and the autonomous replication sequences (ARS) were isolated and used to construct the plasmid that has about 10^5/μg of transformation frequencies and can be maintained as extrachromosomal elements. For example, the plasmid pYM8 is widely used as a shuttle vector in gene complementation studies. *P. pastoris* is amenable for transformation by both the electroporation and spheroplast generation method (Cregg et al., 1985, 1993). A number of genes have been cloned and sequenced in *P. pastoris* due to the availability of a set of auxotrophic strains with essentially wild type genetic background, the plasmids that act as shuttle vectors and the genomic DNA libraries for the isolation of *P. pastoris* genes by functional complementation of mutants. The genes cloned by this method encode a varied set of proteins such as putative ATPase, peroxisomal integral membrane proteins, proteins related to ubiquitin-conjugating enzymes and proteins that might be constituents of the peroxisomal protein import machinery (Nuttley et al., 1995).

Peroxisome assembly factor-1 (PAF1) is a well known protein responsible for the human Zellweger syndrome (Goldfischer, 1996). Dr. Cregg's group has cloned this gene's homolog in *H. polymorpha* (Waterham et al., 1996a, 1996b). Future studies of the function of this gene in yeast will be interesting. It will also be more interesting if we can test if human PAF-1 could

rescue *H. polymorpha* mutant or vice versa and thus to probe the functional conservation in eukayotes. Gould and his coworkers (Dodt et al., 1996) developed a method to isolate human peroxisome biogenesis disorder genes by computer-based homology probing of the dBEST database. They found *PXR1* is a human orthologue of the *P. pastoris PAS8* gene and *PXAAA1* (Yahraus et al., 1996) as a homologue of *P. pastoris PAS5* gene. The same group of Dr. Gould (Kalish et al., 1996) also developed a visual screening method using a peroxisomal form of the green fluorescent protein (GFP). Wild-type cells expressing PTS1-GFP were chemically mutagenized and the mutagenized strains unable to import PTS1-GFP into peroxisomes were identified by fluorescence microscopy. This technique provides an effective visual marker for peroxisomal protein import in living cells. The PTS-GFP containing wild-type cells can be used to regenerate peroxisome mutants.

The available molecular technologies also made *P. pastoris* cells as a host system for the large scale heterologous protein production for industrial usage (Sreekrishna et al., 1997) as well as to attain a certain scale amount of protein for functional study of such proteins. Most of the experiments utilized *AOX1* gene promoter to induce gene expression in *P. pastoris* after *AOX1* gene promoter containing plasmid was introduced into *P. pastoris*. The induction of desired protein synthesis is initiated by methanol addition. The same is true when gene repression is induced by addition of glucose. Dr. Cregg also developed *a P. pastoris* vector containing a promoter of the GAPDH gene

(Waterham et al., 1997). This promoter can induce expression of the gene constructed behind it. This adds convenience for over-expressing the desired protein for observation or other uses. Direct PCR screening of *P. pastoris* clones after yeast transformation to verify the right transformant was also developed recently so that less work is needed to verify the expression of the desired gene in *P. pastoris*.

Previous Research of Peroxisome Degradation in Our Lab

In our lab, we used methanol induction and subsequent glucose or ethanol adaptation to characterize peroxisomal and cytosolic protein degradation pathways in *P. pastoris* (Tuttle et al., 1993, Tuttle and Dunn, 1995). Parental strain GS115 cells under glucose and ethanol adaptation showed different protein degradation approaches biochemically (Fig. 1-2). The activities of peroxisomal alcohol oxidase (AOX) and cytosolic formate dehydrogenase (FDH) were only about 20% of their zero time activity after a 6 hour glucose adaptation. A 6-hour time course of monitoring AOX and FDH activity and the protein level of AOX and FDH in glucose adaptation showed good correlation and verified that the loss of activity was mainly due to loss of AOX and FDH protein (Tuttle and Dunn, 1995). The activities of cytochrome c oxidase (COO), a mitochondria protein and activities fructose-1,6-bisphosphatase (FBP), a cytosolic protein remains high after 6 hour glucose adaptation (data not shown).

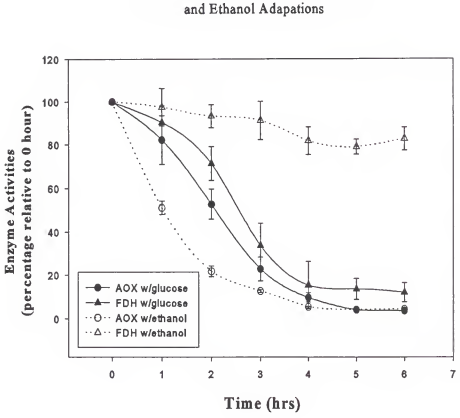


Figure 1-2. Loss of peroxisomal and cytosolic enzymes during ethanol and glucose adaptation. *P. pastoris* (GS115) were cultured in methanol induction medium until stationary, at which time ethanol or glucose were added to begin adaptation (time zero), cell-free extracts were prepared at 0- and 6-hour time point and alcohol oxidase (AOX) and formate dehydrogenase (FDH) activities were assayed. Values are represented as a percentage of the 0-hour activity. All assays were measured at least three times and the values shown on the graph represent the average ± sd.

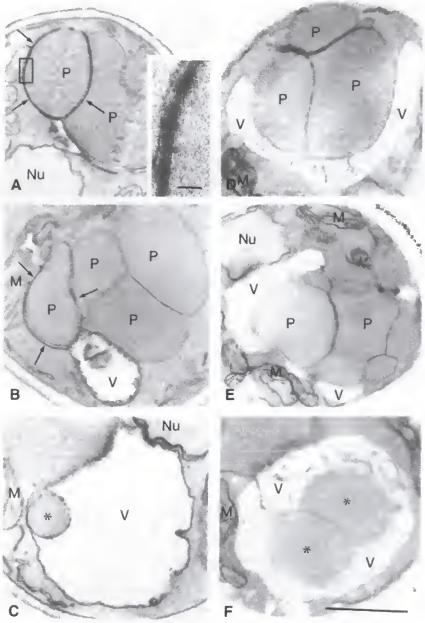


Figure 1-3. Morphological characterization of peroxisomes during glucose and ethanol adaptation. *P. pastoris* cells (GS115) were induced in methanol and then switch to either ethanol (A-C) or glucose (D-F) for one hour. Under glucose adaptation, peroxisomes were engulfed by the yeast vacuole and contents get degraded. However, under ethanol adaptation, peroxisomes were first wrapped by several layers of membrane to form autophagosome, then fused with the yeast vacuole. Bar in panel A inlet: 25nm, Bar in other panels: 0.5μM. (use of this figure is permitted by Dr. D. Tuttle)

Also in the glucose-induced pathway, the degradation process requires protein synthesis since protein synthesis inhibitor cycloheximide prevented peroxisome degradation in GS115 cells. Under ethanol adaptation, AOX activities reduced significantly as it did in glucose adaptation in a 6-hour time course while FDH activity remained unchanged. The degradation of peroxisomes in ethanol-induced pathway in *P. pastoris* is independent of protein synthesis since cycloheximide did not prevent ethanol-induced peroxisome degradation. In the same study (Tuttle and Dunn, 1995), they found proteinases A and B were required for the degradation of peroxisomes and FDH. Peroxisomes in PrA and PrB mutants were induced by methanol. However, during the subsequent adaptation to glucose or ethanol, the mutants showed no significant degradation of AOX and FDH proteins as well as peroxisomes. The combined results suggested that the microautophagy of peroxisomal AOX and cytosolic protein FDH is selective under glucose adaptation condition and regulatory molecules are needed to modulate this process.

The previous observation was also supported by the morphology studies in light microscopy and electron microscopy. Immunofluorescence microscopy studies were used to screen or verify that the degradation process was disrupted in the mutants (see below). Using quinacrine that labels specifically the yeast vacuole, we can follow the generation and degradation of peroxisomes in parental strains and mutants when we combine the observation results of fluorescence microscopy of the yeast vacuole and the phase-contrast

microscope image of yeast peroxisomes. Immunofluorescence microscopy can follow specifically the alcohol oxidase generation and degradation in peroxisomes as well as other proteins that could detected by the antibodies. However, to characterize the specific detailed structures and degradation process of peroxisomes, electron microscopy is needed. As shown in the Fig.1-3, at 1 hour of the glucose adaptation in parental GS115 cells, peroxisomes without additional membrane layers were seen in close association with the yeast vacuole (panel D). The extensions or arms of the vacuole were observed surrounding clusters of peroxisomes (panel E) and the vacuolar membrane protrusions fused to engulf the peroxisomes, a homotypic event of fusion. Peroxisomes were seen being degraded inside the vacuole (panel F). However, after 1 hour of ethanol adaptation, peroxisomes were seen first to be wrapped by several layers of membrane (panel A) to form autophagosomes. The autophagosomes then fused with the yeast vacuole (panel B) and the contents in peroxisome were degraded (panel C). We do not know the origin of these layers of membrane. It is possible that they are synthesized *de novo*. The autophagy pathway under ethanol adaptation is analogous to the macroautophagy that has been characterized in mammalian system (see previous discussion in autophagy section).

Through these observations, we proposed that the microautophagy mechanism of peroxisomes proceeds via a sequence of events: environmental signaling, peroxisomal recognition, peroxisomal sequestration & homotypic

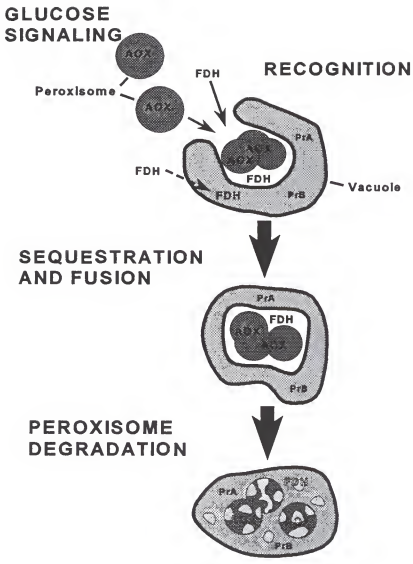


Figure 1-4. Model of glucose-induced microautophagy pathway. The yeast responds to glucose adaptation by degrading peroxisomal alcohol oxidase (AOX) and cytosolic formate dehydrogenase (FDH). Peroxisomes and FDH are recognized by and sequestered within the yeast vacuole. Once within the vacuole, these proteins are degraded by a mechanism dependent upon the actions of proteinases A (PrA) and B (PrB).

vacuole fusion, and vacuolar degradation (Fig. 1-4). We hypothesized that cytosolic, peroxisomal or vacuolar membrane regulatory proteins need to be synthesized during the peroxisome autophagy in yeast cells. The interaction and coordination of these regulatory proteins lead to the degradation of specific proteins or organelles of the cells (Fig. 1-4). To approach this question, Dr. Tuttle has generated glucose-induced selective autophagy (*gsa*) mutants deficient specifically in AOX degradation during the glucose adaptation. Mutants were screened by an colorimetric AOX direct colony assay for their inability to degrade AOX during glucose adaptation and verified by a liquid media AOX assay. We tried to identify molecules regulating the autophagy process as well as to elucidate the mechanisms of the autophagy process. Indeed, in Tuttle's study (Tuttle and Dunn, 1995), he has identified two complementation groups (*gsa1* and *gsa2*) that were defective in the peroxisome degradation pathway during glucose adaptation. The degradation of AOX and FDH in response to glucose was inhibited by 70-90% in both mutants while the degradation of AOX proceeded normally during ethanol adaptation. This suggested that the vacuolar proteinases activities were normal in these mutants and the mutation did not affect vacuolar function but inhibited an event upstream of vacuolar degradation. The ultrastructure studies confirmed that peroxisome entry to the vacuole was defective in these mutants. This also provides further evidence of divergence between these two degradative pathways.

Chapter Summary

The methylotrophic yeast *P. pastoris* readily grows in media containing methanol as the sole carbon and energy source. During this growth stage, they synthesize the large amount of peroxisomal and cytosolic enzymes that are necessary for the utilization of methanol. Upon adaptation to an alternative carbon source such as glucose or ethanol, the peroxisomes are rapidly targeted to and degraded in the vacuole. The peroxisomes are large enough (0.5 to 1 um) to be identified under light and electron microscopy. The degradation process can also be easily detected by a simple colorimetric assay of AOX activities. Based on previous studies in our lab, we hypothesize that the degradation of peroxisomes during glucose adaptation requires regulatory molecules for the signaling, recognition, peroxisomal sequestration & homotypic vacuolar membrane fusion, and degradation steps in the autophagy of peroxisomes. To approach this problem, *gsa* mutants that are defective in AOX degradation during glucose adaptation were generated. I tried to define the molecular events of autophagy in *P. pastoris* by analyzing these mutants. The *Pichia pastoris* is a proven study model for peroxisome assembly, import and peroxisome degradation. The existing knowledge of peroxisome degradation and the available molecular tools combined with biochemical and ultrastructural methods enable us to examine the microautophagy of peroxisomes and to understand the mechanisms of this autophagy in yeast at the molecular level.

CHAPTER 2
MATERIALS AND METHODS

Yeast and Bacterial Strains and Media

All *Pichia pastoris* parental yeast strains were the generous gifts of Dr. J. M. Cregg (Oregon Graduate Institute, Beaverton, OR). The wild type strains, GS115 (*his4*), GS190 (*arg4*), WP1 (*ade4*), WP2 (*met4*) and PPF1 (*his4*, *arg4*), were routinely cultured at 30°C in YPD (1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose). YNM and YND media refer to the growth media containing yeast nitrogen without amino acids plus methanol or glucose respectively. If histidine (40mg/L) is added, the media are called YNMH or YNDH. The sporulation and mating medium (SM media) is composed of 0.5% sodium acetate, 1.0% KCl, 1.0% glucose and 2.0% agar. The electroporation medium is composed of 1M Sorbitol, 2% glucose, 0.1% yeast nitrogen base (YNB), 0.4 mg/L biotin and 2% of agar. Bacto peptone, yeast extract and media agar were purchased from Fisher Co. LB broth media is composed of 0.5% yeast extract, 1% tryptone, 0.5% NaCl. Ampicillin is added to LB media at a final concentration of 100 µg/ml when selection is needed for *E. coli* growth. WDY (*gsa*) mutants were generated by Dr. Dan Tuttle by selecting

Table 2-1 ***P. pastoris*** **Strains Available for Current Studies**

Strain(Species)	Complementation group	Genotype
GS115	Parental strain	his4
GS190	Parental strain	arg4
WP1	Parental strain	ade4
WP2	Parental strain	met4
KB1	Parental strain	ade4, his4
JC205	Parental strain	met4, his4
PPF1	Parental strain	his4, arg4
WDY1(12.1)	*gsa2-1*	his4, gsa2-1
WDY2(4.3)	*gsa1-1*	his4, gsa1-1
WDKO1	*Δpfk1*	pfk1::ARG4, his4
WDY3(13.1)	*gsa1-2*	his4, gsa1-2
WDY4(18.1)	*gsa4*	his4, gsa4
WDY5(6.3)	*gsa5*	his4, gsa5
WDY6(4.1)	*gsa6*	his4, gsa6
WDY7(13.2)	*gsa7*	his4, gsa7
WDKO7	*Δgsa7*	gsa7::ARG4, his4
WDY8(17.3)	*gsa1-3*	his4, gsa1-3
WDY9(15.2)	*gsa?*	his4, gsa?
WDY10(35.3)	*gsa8*	his4, gsa8
WDY11(35.2)	*gsa?*	his4, gsa?
WDY12(9.3)	*gsa?*	his4, gsa?
WDY13(32.1)	*gsa3*	his4, gsa3
WDY14(15.1)	*gsa?*	his4, gsa?
WDY15(18.3)	*gsa?*	his4, gsa?
WDY16(32.2)	*gsa?*	his4, gsa?

glucose-induced selective autophagy deficient strains in chemically mutagenized parental GS115. WDKO1 (*Δpfk*1) is a *PFK1* gene knockout strain derived from PPF1 by replacing its *PFK1* gene with a *S. cerevisiae ARG4* gene. WDKO7 (*Δgsa7*) is a knockout strain of *GSA7* from PPF1. Both *Escherichia coli* DH5α and Epicurian coli® XL1-blue (Stratagene, Co.) were used to amplify plasmids. A list of yeast strains used in the study is included in the Table 2-1.

Enzyme Assays

Yeast cells were first cultured in the methanol induction medium. Glucose or ethanol was then added for adaptation. The yeast cells were harvested at stationary stage (zero hour time point of adaptation) and then six-hours after adaptation (six hour time point of adaptation). Two ml of yeast cells were pelleted at 4°C, 2500 rpm. The cells were resuspended in 1 ml ice cold breaking buffer (20 mM Tris/Cl, pH 7.5, 50 mM NaCl, 1mM EDTA). Cells were broken by vortexing 1 min for 3 times mixed with 500μm diameter glass beads (Tuttle et al., 1993). Phenylmethylsulfonyl fluoride (PMSF, 1mM) was added to prevent proteolysis. After centrifugation at 4°C, 2500 rpm for 5 min, the supernatant was aspirated and stored at 4°C for subsequent enzyme assays.

Alcohol oxidase activity was measured in a reaction coupled with horseradish peroxidase and the oxidation of 2,2'-azino-bio(3-ethylbenz-thiazoline-6-sulfonic acid), ABTS. The reaction mixture contained horseradish

peroxidase and ABTS in 33 mM potassium phosphate buffer (Tuttle et al., 1993). The samples were incubated at 37°C until green color developed. The color was measured at 410 nm. The activity of formate dehydrogenase was determined by measuring the formation of NADH during the oxidation of formate by absorption at 340 nm (Kato, 1990). The reaction mix was made of 33 mM potassium phosphate buffer, pH 7.5, 2 mM NAD^+, 167 mM sodium formate per reaction plus cell free sample. The rate of change of absorbance at 340nm was followed and used as a measure of enzyme activity.

Phosphofructokinase activity and other glycolysis enzyme activities were measured in a coupled enzyme assay resulting in the oxidation of NADH/ NADPH or reduction of NAD/NADP. The NADP and NADH cocktail solution for enzyme activity assays were composed of 98 mL 50mM triethanolamine, 10mM $MgCl_2$ pH7.4, 2 mL NADP 14mg/mL or 0.75 mL NADH (30 mg/mL in 0.1 M Tris pH 7.6) respectively. Other substrates and enzymes were added according to the reaction requirement and finally extracts were added. The activities recorded were adjusted by the extract protein concentration. For example, hexokinase was assayed by a cocktail containing NADP, 0.1 M fructose, 1mg/mL phososphoglucose-isomerase, 1mg/mL of glucose-6-phosphate dehydrogenase, yeast cell extract, and the reaction was started with 0.1 M ATP. The production of NADPH was monitored by a kinetic program with an absorbance reading set at 340nm. Pyruvate kinase was assayed by a cocktail containing NADH, 0.1 M ADP, 0.1 M fructose-1,6-bisphosphate, lactate dehydrogenase, the cell free

extract and beginning the reaction by addition of 0.1 M Phosphoenolpyruvate. The production of NAD was measured by a kinetic program at OD 340nm (Dyson et al., 1975). The glycolysis enzymes tested were GK: glucokinase; HK: hexokinase; PGI: phosphoglucose isomerase; PFK: phosphofructokinase; ALD: aldolase; TIM: Triosphosphate isomerase; PGM: phosphoglycerate mutase; ENO: enolase; and PK: pyruvate kinase. Protein concentrations were measured using crystalline bovine serum albumin as a standard (Bradford, 1976).

Isolation of Glucose-Induced Selective Autophagy-Deficient (*gsa*) Mutants

Mutant Generation and Isolation

WDY mutant strains were generated by mutagenizing parental GS115 cells with nitrosoguanidine at 100 µg/ml concentration (Tuttle and Dunn, 1995). The mutagenized cells were screened for the loss of the ability to degrade alcohol oxidase in response to a shift in a carbon source from methanol to glucose with a direct colony assay (Tomlison, 1992, Tuttle and Dunn, 1995). Briefly, the mutagenized cells grown on YPD plates were replica-plated to YNMH plates (6.7 g/L yeast nitrogen base without amino acids, 0.5% methanol, 0.4 mg/L biotin, and 40 µg/ml histidine) and the colonies were allowed to grow for 4-5 days. The colonies were replica-plated onto nitrocellulose and placed on YNDH plates (6.7 g/L yeast nitrogen base without amino acids, 2% glucose, 0.4 mg/L biotin, and 40 µg/ml histidine) for 12-16 hours. Those putative mutant

colonies which retained AOX despite glucose adaptation were identified by the purple color reaction product of the direct colony assay (Tuttle and Dunn, 1995). The principle of reaction is the same as the alcohol oxidase assay. Those mutant strains appeared purple were isolated and their inability to degrade AOX during glucose adaptation verified in liquid cultures. This was done by first growing the cells for 24-36 hours in 20 mls of methanol induction medium that consisted of 6.7 g/L yeast nitrogen base without amino acids (Difco), 0.4 mg/L biotin, 40 µg/ml L-histidine, and 0.5% methanol. Glucose was then added to a final concentration of 2% at the beginning of glucose adaptation. Cells were harvested at 0h and 6h of glucose adaptation and lysed by vortexing with acid-washed glass beads. The resulting homogenates were then assayed for AOX activity according to the procedures of Tuttle and Dunn (1995).

Mutant Backcrossing

To further characterize the mutant strains, *gsa* mutants that have been identified were backcrossed to GS190 (*arg4*) and GS115 (*his4*) for generating *gsa* specific mutants as well as for identifying complementation groups. The haploid mutants were sequentially mated to essentially wild type strains (GS190 and GS115) containing complementary auxotrophic markers and haploid progeny recovered. Several rounds of backcrossings resulted in a particular mutant strain with a mutated gene of our interest. One round of backcrossing was accomplished as follows: the mutant strains were streaked onto YPD plates in patches and the parental strains of the complementary auxotrophy was spread

on YPD plates to form a lawn of cells and both plates were incubated at 30°C overnight. The lawn and the patch were both replica plated to a single sporulation media (SM) plate and incubated overnight at 30°C to induce mating. These plates were then replicated to YND plates without amino acids and incubated for 2 to 3 days until diploid colonies appeared. Colonies from these plates were streaked onto a fresh YND plates and incubated for 1 to 2 days and then were streaked on YPD plate. Cells from this plate were then streaked to SM plates and incubated four days at 30°C to induce meiosis and sporulation. Cell spores were harvested with an inoculation loop and the remaining diploid cells were killed by etherization. An aliquot from this mixture was diluted 1 to 100 and 100 µl plated on YPD plates for two days for the haploid cells to grow. The cells were then washed off and plated on YNMH or YNMA plate for a direct colony assay for mutants screen. The verified mutant via this assay and the subsequent AOX liquid media assay was selected and used for the next round of backcrossing with a parental strain that has the opposite auxotroph.

Mutant Complementation Analysis

To determine the number of defective genes present in the *gsa* mutants in my possession, the complementation groups of *gsa* mutants had to be identified first. To achieve this, different auxotroph strains containing mutants (for example, a histidine auxotroph and an arginine auxotroph mutant) are mated and a prototroph (diploid) of them is generated. The ability of this diploid to degrade peroxisomes can be tested in a glucose adaptation assay. If the

peroxisome degradation can proceed in this diploid, these two strains are said to complement each other. Complementation indicates the defective genes in these two mutants are different and the mutants belong to different complementation groups. If the diploid still could not degrade peroxisomes, the two mutant strains presumably contain different mutant alleles of the same gene and they belong to the same complementation group.

Molecular Biology

Yeast Transformation

The genomic library was a gift of Dr. J.M. Cregg (Oregon Graduate Institute). It was constructed by a partial digestion of genomic DNA with Sau 3A I first. The digestion products were then run on a DNA gel and the DNA of 5-10 kb size was collected, purified and ligated into BamH I site of plasmid pYM8. *Pichia pastoris* cells (*his4*) were grown in YPD to an OD600nm = 1.0 harvested, and transformed by electroporation at 1.5 kV, 25 μF, 400 Ω (Gene Pulser, Bio-Rad Corporation) with 5 to 10 μg of genomic DNA library. The transformed cells were grown for 4-5 days on electroporation plates, and the colonies were replica-plated to nitrocellulose and placed on YND-aa plates for 12-16 hours. Those colonies which appeared purple or white (according to experiment purposes) upon a direct colony assay were isolated and their abilities to degrade AOX during glucose adaptation verified in liquid media as described above.

Stable transformation with vectors pYM8 and pYM4 containing specific fragments of genomic DNA was also done by electroporation. pYM8 was derived from pBR322 and contains the 164 bp autonomous replicating sequence from *P. pastoris* and the *HIS4* gene of *S. cerevisiae*. pYM4 was also derived from pBR322 and contains a *HIS4* gene of *P. pastoris*, but lacking an autonomous replicating sequence. pYM25 was derived from pBR322 and contains an *ARG4* gene of *S. cerevisiae*. Both pYM4 and pYM25 are non-episomal vectors. Stable integration of the episomal pYM8 constructs was accomplished by two cycles of growth under nonselection and selection conditions. Stable integration of non-episomal pYM4 or pYM25 constructs was promoted by a single cut with restriction enzyme Stu I within the *HIS4* locus. This cut should also direct the integration into His4 locus in the transformed cell genome. The plasmid maps of pYM8 and pYM4 is shown in Fig. 2-1.

Plasmid Isolation and DNA Sequencing

The rescued colonies that had been complemented with the genomic DNA library were grown overnight in 2 ml of YPD medium. The cells were pelleted and resuspended into 0.2 ml of 2% Triton X-100, 100 mM NaCl, 1 mM EDTA, 1% SDS, and 10 mM Tris/HCl, pH 8.0 and 0.2 ml of phenol:chloroform: isoamyl alcohol (25:24:1). The cells were then disrupted by vortexing in the presence of 0.5 ml acid-washed glass beads. After centrifugation, 2 μl of the upper phase containing the plasmid was used to transform *E. coli* DH5α cells (Ausubel et al., 1988). The plasmid was then isolated by Wizard *Plus* minipreps

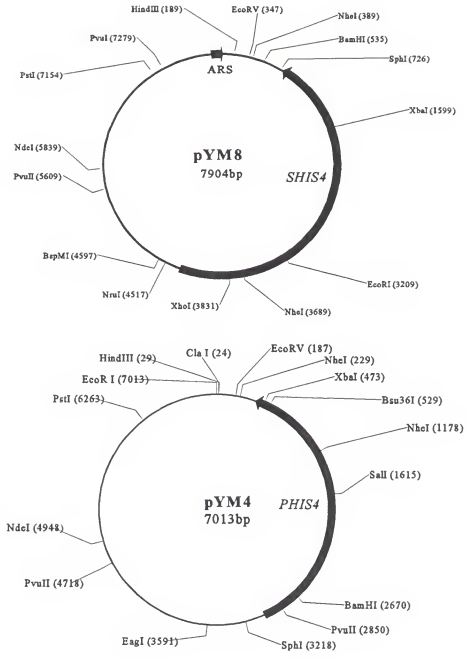


Figure 2-1. Maps of plasmids pYM8 and pYM4. pYM8 is constructed based on pBR322 with a *S. cerevisiae HIS4* gene and an autonomous replication sequence (ARS). pYM4 is also based on pBR322 but lacking an ARS. pYM4 contains a *P. pastoris HIS4* gene. The main restriction enzyme sites are also indicated in the two plasmid maps.

of Promega and restriction analysis was done to identify the genomic insert size and sites. The genomic DNA fragment was excised from the pYM8 shuttle vector and engineered into pBluescript/KS for sequencing by nested deletion (Ausubel et al., 1988). Direct sequencing of pDLT1 was also done using self generated primers that flank the desired region. The primers for sequencing were synthesized by DNA synthesis lab, University of Florida. All sequencing was performed by University of Florida Sequencing Core via the dideoxy chain termination method. The insert of pDLT1 was sequenced from both plus/minus strands. The DNA sequences were assembled in DNAman® or Gene Runner® programs and all six reading frames were compared to protein sequence databases using the Blastx program of the National Center for Biotechnology Information (NCBI).

Northern, Southern and Western Blot Analysis

Yeast cells in exponential growth were pelleted and resuspended in 1% SDS, 10 mM EDTA, and 50 mM sodium acetate, pH 5 solution. Total RNA was extracted with addition of hot phenol/chloroform/ isoamyl alcohol (25:24:1). About 10 µg of total RNA was loaded on glyoxal agarose gels. The RNA was then transferred to Maximum Strength Nytran® (Schlelcher & Schuell Inc.) by overnight blotting (Ausubel et al., 1988). The RNA in the blot was cross-linked by UV for 12 seconds and then subjected to methylene blue staining for 2 min. The staining pattern of ribosomal RNA was photographed or scanned as loading control. A 2.5 kb DNA fragment in *PFK1* gene was used as template for ^{32}P-

labeling by random priming reaction with NEB's NEBlot™ Kit. The probe was cleaned by NucTrap® Push Columns (Stratagene Inc.). Northern blotting using labeled DNA probes were done as described (Ausubel et al., 1988). An equivalent 10 million counts of radiolabeled DNA probes were used for one typical labeling.

For Southern blot purpose, genomic DNA was isolated from stationary growth cells. It was then digested with BamH I, separated on agarose gels, and then transferred to nylon (Kaiser et al., 1994). ^{32}P-labeled DNA probe labeling was done as described in Northern blotting.

To prepare Western blot samples, *P. pastoris* were grown on YPD or on methanol and then adapted to glucose. The yeast cells were collected and prepared for SDS gel according to Kaiser (Kaiser et al., 1994). The samples were separated on a SDS minigel (Bio-Rad). The proteins were transferred to the nitrocellulose and incubated with anti-HA.11 mAb (against mouse) or polyclonal antibody (against rabbit) (Babco). The blot were then incubated with secondary anti-mouse or anti-rabbit antibodies conjugated with HRP. Protein bands was detected by ECL method (Amersham).

Isolation of *PFK1* and *GSA7* Knockouts

The construction of WDKO1 (*his4*, *pfk1*::*ARG4*) cell was done by disrupting the *PFK1* gene of PPF1 (*his4, arg4*). First, the 1.2 kb Hind III fragment within the open reading frame of *P. pastoris PFK1* was replaced with the 3.0 kb *S. cerevisiae ARG4* gene from pYM25. A linear 5.6 kb fragment with

the *ARG4* gene flanked by *PFK1* was excised from the shuttle vector by BamH I digestion and used directly to transform PPF1 cells. Cells were grown on transformation plates supplemented with histidine (40 µg/ml). Colonies were isolated and their ability to degrade AOX during glucose adaptation examined (see Mutants Generation and Isolation section). *PFK1* gene disruption was verified by measuring phosphofructokinase activity assay (Blangy et al., 1968) and by Southern blotting (see above). The construction of WDKO7 was basically the same as for WDKO1. The *S. cerevisiae ARG4* gene was put in between Hind III and Bgl II sites of the *GSA7* gene and the knockout fragment was cut out by Apa I and Sca I restriction enzymes and used to transform PPF1 cells. The transformants were first screened with an AOX direct colony assay. The colonies that appeared purple in the assay should lose AOX degradation ability and are thus the putative "knockouts". Possible knockouts were tested for the correct gene targeting by PCR the genomic DNA extracted from these clones. The clone has the expected PCR fragment (4.9 kb instead of 2.5 kb in a normal strain) is the true knockout and was named as WDKO7 *(Δgsa7)*.

Site-directed Mutagenesis of *PFK1*

The aspartic acid at position 362 of Pfk1 protein was changed to a serine by site directed mutagenesis. This was done using the Stratagene® QuikChange™ Site-Directed Mutagenesis Kit. Mutated *pfk1* was prepared by PCR utilizing two complementary oligonucleotide primers (synthesized at the University of Florida Oligonucleotide Core) that contained the desired two base

mutations, *Pfu* DNA polymerase, and pDLT1 containing the wild type *PFK1* as a template. This resulted in "nicked" plasmid containing *pfk1*. The template DNA was digested by *Dpn* I endonuclease and the nicked plasmid was repaired and amplified in Epicurian Coli® XL1-Blue cells. *Pfk1* was then subcloned into pYM4 (pWP-pfk). Sequencing in both directions of the pWP-pfk was performed at the University of Florida Sequencing Core by the dideoxy chain termination method and desired bases change was verified (see Fig. 5-4). pWP-pfk was then introduced into *gsa1-1* and WDKO1 by electroporation. The transformants were selected based on the integration of *HIS4* gene of plasmid into the genome. The linearization of the plasmid inside *HIS4* gene promoted integration into *HIS4* gene locus of the genome.

Generation of a HA Epitope Tag in *GSA7* and *YHR171w* Gene

Two primers were designed to PCR the *GSA7* gene plus a HA epitope tag at the C terminus of the protein. The 5' primer started 350 bases before the start codon of *GSA7* in order to include its endogenous promoter, while the 3' primer was at the end of the coding sequence plus a HA epitope tag. The HA epitope tagged *GSA7* gene was amplified by PCR with ID-PROOF™ polymerase (ID Labs Biotechnology). The successful construct was verified and subcloned into vector pYM4 Cla I and EcoR V sites (pYWP7-HA). The construct pYWP7-HA was linearized in *HIS4* gene or *GSA7* gene and used to transform *gsa7* cells. In *S. cerevisiae*, the same strategy was used to generate a HA epitope tagged *YHR171w* and put into pYM4. The *YHR171w* gene was also put in the pHWO10

vector behind a GAP promoter (Waterham et al., 1997) and in the pPIC3 vector behind an AOX promoter (both vectors are a gift of Dr. JM Cregg). These constructs were used to transform *S. cerevisiae* as well as *P. pastoris*. The expression of HA tagged protein was confirmed by Western blot by HA antibodies.

Fluorescence Microscopy and Electron Microscopy

Yeast cells were grown on YPD, glucose, ethanol or methanol media for different experiment setting. The cells were then fixed in 3% (w/v) of paraformaldeyde (pFA) for 10 min at room temperature. The yeast cells were centrifuged and resuspended in 50mM potassium phosphate buffer (Kpi) / 3% pFA at room temperature for another 30 min with gentle rotation. The cells were washed with Kpi and resuspended in SPC buffer (50 mM K_2HPO_4, 16 mM citric acid, 1M sobitol, pH 5.8). The cells were spheroplasted with Zymolase 20T in SPC buffer for 15 min and the digestion was monitored every 5 min for cell wall digestion completion. After digestion, the cells were washed with SPC buffer and put on a 15-well slide precoated with polylycine (ICN Biomedicals). The fluorescence containing media such as phalloidin, quinacrine were applied to the slide for direct observation or the slides were placed in the blocking medium for immunofluorescence microscopy study (5% normal goat serum in PBS). For this purpose the cells on the wells were incubated with primary antibody for 1-2 hr or

overnight and then with secondary antibody for one hour. The observation was done with a Zeiss axiophot photomicroscope using a Zeiss 100X oil emersion.

Ultrastructural analysis was performed using a potassium permanganate fixation protocol (Veenhuis et al., 1983). This procedure effectively delineates membrane structures in methylotrophic yeasts. Briefly, cells were harvested by centrifugation, washed in water, and fixed in 1.5% $KMnO_4$ in veronal-acetate buffer (0.3 mM sodium acetate; 0.3 mM sodium barbital, pH 7.6) for 20 min at room temperature. The specimens were then washed three times and dehydrated in increasing concentrations of ethanol wash. This was followed by 100% propylene oxide twice. The cells were then infiltrated with a 50:50 mix of propylene oxide and the POLY/BED 812 (Polysciences, Inc., Warrington, PA) for two days. The preparations were dried by a vacuum overnight, infiltrated with 100% POLY/BED with accelerator 2,4,6-Tri(dimethylaminomethyl) phenol (DMP-30®, Polysciences, Inc.) for another two days, and then incubated in an oven overnight at 60°C. The resulting samples were then mounted on the blocks and the blocks were then sectioned (by D. Player, Department of Anatomy and Cell Biology, University of Florida College of Medicine) and prepared for examination on a JEOL 100CX II transmission electron microscope.

CHAPTER 3
CHARACTERIZATION OF *PICHIA PASTORIS* MUTANTS DEFECTIVE IN GLUCOSE-INDUCED MICROAUTOPHAGY

Introduction

Methylotrophic yeast *P. pastoris* can utilize methanol as a sole carbon source by synthesizing those peroxisomal and cytosolic enzymes necessary to assimilate methanol. When *P. pastoris* cells were grown on methanol, their peroxisomes were induced in large size and numbers. During the subsequent adaptation to glucose or ethanol, their peroxisomes were rapidly degraded (Tuttle et al., 1993). *P. pastoris* has been an ideal model to investigate the molecular events of peroxisome biogenesis (Subramani, 1996) as well as peroxisome degradation (Tuttle and Dunn, 1995).

Our lab has identified two distinct pathways for the degradation of peroxisomes in *P. pastoris* (Tuttle and Dunn, 1995) namely, microautophagy and macroautophagy. These pathways are independently regulated, but share at least one common event, vacuolar degradation. In order to better define the molecular aspects of peroxisome turnover in *P. pastoris*, glucose-induced selective autophagy deficient (*gsa*) mutants have been generated from the

Steps for the Identification of those Proteins Required for Microautophagy

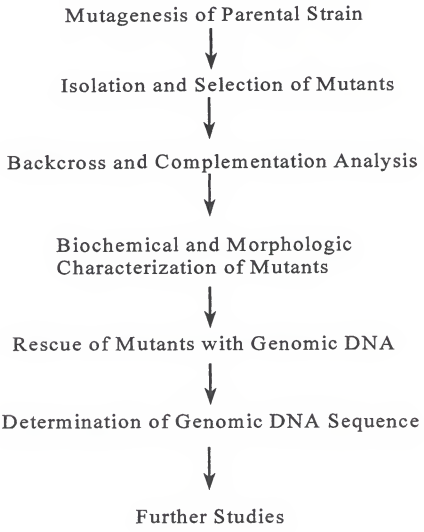

Mutagenesis of Parental Strain
Isolation and Selection of Mutants
Backcross and Complementation Analysis
Biochemical and Morphologic Characterization of Mutants
Rescue of Mutants with Genomic DNA
Determination of Genomic DNA Sequence
Further Studies

Figure 3-1. A flow chart of mutants characterization. A mutant was selected for its inability to degrade AOX during glucose adaptation. Once its biochemical and morphological profile had been identified, it went through backcrossing and complementation analysis. The mutant was then transformed with a *P. pastoris* genomic DNA library to identify the responsible gene. Once the gene was recovered and verified, the gene was sequenced and, according the sequencing result, the subsequent studies were determined and carried out.

parental strain GS115 by chemical mutagenesis (Tuttle and Dunn, 1995) for their inability to degrade peroxisomal alcohol oxidase (AOX). The putative mutants that appeared purple in an AOX direct colony assay were selected. They were then subjected to liquid media AOX and FDH assays for further verification. The mutant strains were then backcrossed with parental GS190 (an arginine auxotroph) and then mated with another mutant in a histidine auxotrophic background (see below), to identify their complementation groups. The selected mutants were also backcrossed with parental GS190 and GS115 two to four times to eliminate background mutations that were generated in the chemical mutagenesis unrelated to *gsa* phenotype. A flow chart of mutant generation, identification and characterization steps has been included in Fig. 3-1. In this chapter, I will detail the genetic, biochemical and morphological characterization of *gsa* mutants.

Complementation Group Identification

The purpose of the complementation analysis of mutant strains is to provide an indication of the genetic relationship of the mutations involved and thus to further characterize the *gsa* mutants. Complementation groups were identified by backcrossing different *gsa* mutants with an arginine auxotroph GS190. The resulting arginine mutants were then mated with other *gsa* mutants (histidine auxotrophs) as well as parental GS115. Their progeny were analyzed

for their peroxisomal AOX degradation ability. If the progeny (diploid cells) rescued the defect of both mutant strains (regaining AOX degradation ability), then these two strains represent two different complementation groups and two unique genes in each of the mutants is defective. If they complemented each other, they belong to the same complementation group with possible different mutated alleles. A sample complementation study graph of strain WDY4, WDY8 and WDY10 is shown in Fig. 3-2. AOX activities of six hour glucose adaptation were presented as a percentage of zero hour activities. As shown in the graph, the progeny of WDY8 and WDY4 could degrade AOX and little AOX activity remained after 6 hour glucose adaptation, and thus they complemented each other. WDY4 and WDY8 did not complemented WDY10. The same was true for WDY10 and GS115. The result of AOX activity assays of these diploids clearly showed that WDY4, WDY8 and WDY10 are three separate *gsa* groups. The fact that WDY10 was not complemented by GS115 made it clear that WDY10 has a dominant trait. Currently, I have identified eight complementation groups. All *gsa* mutants except *gsa8* showed recessive traits while *gsa8* showed a dominant trait. WDY2, WDY3 and WDY8 belong to the same *gsa1* mutant group and are thus named as *gsa1-1, gsa1-2* and *gsa1-3* respectively. A list of *gsa* mutants is shown in Table 3-1. Some of the mutants showed variable AOX degradation results, such as WDY9, 11, 12, 14 and WDY15. This variability may be due to several mutations in one single mutant strain and thus this strain might need to be backcrossed before complementation analysis. Since there

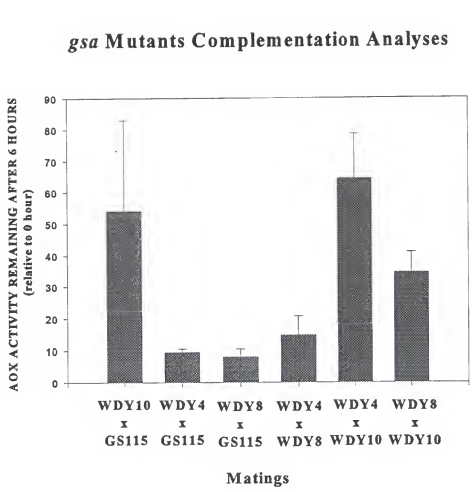


Figure 3-2. Identification of *gsa* mutants complementation groups. The figure is used to illustrate how complementation groups were identified. WDY4, WDY8 and WDY10 were screened from frozen stocks and identified as *gsa* mutants. They were then subjected to backcrossing. The resultant WDY8a1, WDY10a1, WDY4a1 (Arg auxotroph, generation one) were then mated with GS115 cells and themselves. The progenies of mating were subjected to AOX assay. Values of AOX activities after 6 hour glucose adaptation were represented as a percentage of the 0-hour activity. They were measured at least three times in the separate experiments and were shown on the graph as average ± sd. The result showed that WDY4, WDY8 and WDY10 belong to three new complementation groups. However, WDY10 showed a dominant trait since the prototroph of WDY10 and other mutants as well as GS115 showed no rescue effect. WDY4 and WDY10 were subsequently named as *gsa4* and *gsa8*. WDY8 was subsequently found to be in the same *gsa* group as *gsa1-1* (data not shown) and was thus named as *gsa1-3* (WDY3 has been identified as *gsa1-2*).

are still more potential mutants and their complementation groups have not been identified, more work needs to be done on the remaining mutants. Although the identified eight *gsa* complementation groups cover only part of the regulatory molecules that control the process of autophagy, further characterization of these mutants now is possible because of the establishment of these complementation groups. Future studies of the remaining mutants will yield information to better cover all the molecules participating in the peroxisome degradation process as well as to better understand the autophagy mechanism.

Screening for *gsa* Mutants

At the time of complementation analysis of *gsa* mutants, the *gsa* mutants were also going through backcrossing cycles (see Materials and Methods for backcrossing steps). The purpose of backcrossing was to prepare *gsa* mutants for further genetic study. Because the mutant strains were generated by random chemical mutation, the mutations occurred at various sites in the genome. By backcrossing two to four times back & forth from histidine auxotroph background to arginine auxotroph background, most background mutations that were not related to *gsa* phenotype were eliminated.

In eight *gsa* complementation groups (*gsa1* to *gsa8*) (see table 2-1) that have been identified, *gsa1-1* and *gsa2* have been successfully backcrossed four times. *Gsa3, gsa5, gsa7* and *gsa8* have been backcrossed two times and *gsa4*

has been backcrossed one time, but the phenotype of this strain is not stable in its arginine auxotroph background. *Gsa6* has a stable and strong phenotype biochemically. However, we have failed in three separate attempts to backcross it. Once it was placed into the arginine auxotroph background, the phenotype was lost. It is possible that the phenotype effect of *gsa6* is an accumulated one of several defective genes. Backcrossing that leads to the loss of any one of them shows no phenotype in *gsa6*. *Gsa1-1* and *gsa7* were later chosen for further genetic analysis.

Glucose Induced Microautophagy is Defective in *gsa* Mutants

Previously, we have shown that parental strain GS115 undergoes microautophagy during glucose adaptation. The loss of AOX activity, the degradation of AOX protein and the degradation of peroxisomes were compatible (Tuttle and Dunn, 1995). We selected *gsa* mutants on the basis that the mutant could not degrade AOX and peroxisomes. Of the collection of possible mutants, I have identified eight *gsa* complementation groups. All mutants were unable to degrade peroxisomal AOX during glucose adaptation while their ethanol-induced macroautophagy proceeded normally (Fig. 3-3 and Fig. 3-4). The biogenesis of peroxisomes observed during methanol induction was intact in all these mutants as determined by light microscopy. The degradation of AOX and FDH during glucose adaptation was rapid in parental

GS115 cells (also see Fig. 1-2). Less than 20% of the activities of AOX and FDH was left in GS115 after six hour glucose adaptation. However, in mutants presented in these two graphs, they lacked the ability to degrade AOX that has been induced under methanol growth condition. Compare to parental GS115 cells, AOX degradation in response to glucose adaptation in all mutants was inhibited to various extents. The degradation of FDH, a cytosolic protein that required for methanol utilization, was also shut down in most *gsa* mutants. However, in *gsa4* and *gsa8*, FDH degradation proceeds nearly normal when compared with parental GS115. Since FDH is a cytosolic protein, one possibility is that a mutation block an early event of peroxisome microautophagy pathway in this mutant but the early cytosolic protein degradation pathway may be controlled differently from that of peroxisome degradation and thus the FDH degradation process is intact. Interestingly, in the complementation analysis done by Mr. Kendal (Kendal, unpublished data, 1993), *gsa4* strain did not complement either *gsa1-1* or *gsa2* cells. This may be either due to more than one mutant gene in it since *gsa4* was not backcrossed at the time of experiment or to some unknown reasons. As for *gsa8* cells, I found it is a dominant mutant in our complementation analysis. We still need to define the relationship of dominant *gsa8* and FDH degradation.

Ethanol induced peroxisome degradation in all mutants proceeded normally (see Table 3-1). This confirmed that our *gsa* mutants only affected early events of microautophagy of peroxisomes since vacuolar degradation of

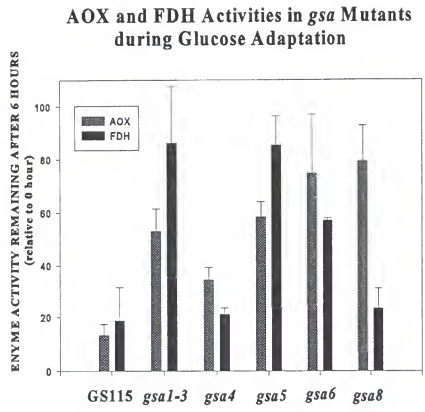


Figure 3-3. AOX and FDH activities of *gsa* mutants under glucose adaptation. *P. pastoris* (GS115) and *gsa* mutants were cultured in methanol induction medium until stationary, at which time glucose was added to begin adaptation (time zero). Cell-free extracts were prepared at 0- and 6-hour time point and alcohol oxidase (AOX) and formate dehydrogenase (FDH) activities were measured. GS115, *gsa1-3*, *gsa4*, *gsa5*, *gsa6* and *gsa8* are included in this figure. Values are presented as a percentage of the 0-hour activity. All assays were measured at least three times and the values shown on the graph represent the average ± sd.

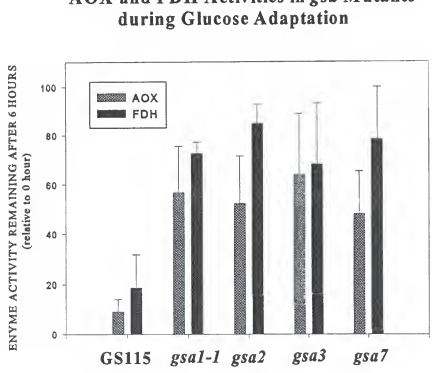


Figure 3-4. AOX and FDH activities of *gsa* mutants under glucose adaptation. *P. pastoris* (GS115) and *gsa1-1, 2, 3* and *gsa7* were cultured in methanol induction medium until stationary, at which time glucose was added to begin adaptation (time zero). Cell-free extracts were prepared at 0- and 6-hour time point and alcohol oxidase (AOX) and formate dehydrogenase (FDH) activities were measured according to Materials and Methods. Values are presented as a percentage of the 0-hour activity. All assays were measured at least three times and the values shown on the graph represent the average ± sd.

Table 3-1 Characterization of *gsa* Mutants

Strains	*gsa* group	Genotype	Phenotype			
			Glucose		ETOH	
			AOX	FDH	AOX	FDH
GS115	Parental strain	*his4*	+	+	+	-
GS190	Parental strain	*arg4*	+	+	+	-
PPF1	Parental strain	*his4, arg4*	+	+	+	-
WDY1	*gsa2*	*his4, gsa2*	-	-	+	-
WDY2	*gsa1-1*	*his4, gsa1-1*	-	-	+	-
WDY3	*gsa1-2*	*his4, gsa1-2*	-	-	+	-
WDY4	*gsa4*	*his4, gsa4*	-	+	+	-
WDY5	*gsa5*	*his4, gsa5*	-	-	+	-
WDY6	*gsa6*	*his4, gsa6*	-	-	+	-
WDY7	*gsa7*	*his4, gsa7*	-	-	+	-
WDY8	*gsa1-3*	*his4, gsa1-3*	-	-	+	-
WDY10	*gsa8*	*his4, gsa8*	-	+	+	-
WDY13	*gsa3*	*his4, gsa3*	-	-	+	-

peroxisomes was intact in ethanol induced macroautophagy which is shared with microautophagy. Although my work focused on glucose-induced microautophagy, the studies of ethanol-induced macroautophagy are interesting and important. This is because most of autophagy mutants in *S. cerevisiae* and *H. polymorpha* are macroautophagy mutants. Only *P. pastoris* can undergo both degradation pathways during glucose and ethanol adaptation. A list of complete biochemical profiles of *gsa* mutants and parental strains are compiled in Table 3-1. GS115 is the parental strain used for chemical mutagenesis. GS190 is used for backcrossing. PPF1 is a double auxotroph used later in the experiment. Their biochemical profiles are provided. All identified *gsa* mutants are included in the table. Some of them are not listed here due to the variable results of their biochemical studies such as WDY9, 11, 12, 14 and WDY15.

While I was studying *gsa1-1*, I also backcrossed WDY1 (*gsa2*) four times to prepare for further genetic analysis. After I finished biochemical and morphological studies of *gsa2*, I transformed *gsa2* cells with a genomic DNA library to identify *GSA2* gene. The colonies appeared white in an AOX direct colony assay regained their AOX degradation ability and thus should bear rescue plasmids with *GSA2* gene. Much to my surprise, about 10% of the colonies appeared white on the AOX direct colony assay and thus were putative rescue clones. The positive and negative control strains tested in the direct colony assay worked fine which meant the detection of white rescue colonies was not due to technical errors. I eliminated some of the false positive rescue

colonies by using liquid media AOX assay. However, I still have too many promising colonies. Twelve plasmids were recovered from 12 of these colonies. Each plasmid appeared different based on restriction enzyme analysis. Some of them had no genomic DNA insert but still rescued *gsa2*. Although I could not exclude the possibility that there are *GSA2* or *GSA2* suppressor genes in my collections of "rescue colonies", I could not identify them.

I tested different growth conditions in order to seek optimal conditions to grow and to characterize the rescue clones. One surprising phenomenon was that the *gsa2* mutant was rescued by addition of 80 µl of 10mg/mL histidine at the beginning of glucose adaptation during liquid media AOX assay. Subsequent studies verified that this was a true phenomenon and the doubling of histidine amount at the beginning of methanol induction did not affect this result. This fact promoted me to re-consider the failed *gsa2* rescue. In the rescue study, only mutants bearing pYM8 containing plasmids and expressing histidine could survive and grow. Since *gsa2* can be complemented by histidine, the expression of histidine in the complemented strains partially or completely offset the phenotype of *gsa2* and thus resulting in a lot of rescue colonies. The expression level of histidine might be quite different in each clone and thus not all strains bearing pYM8 could rescue *gsa2*. We do not know why histidine rescues *gsa2* when it was added upon glucose adaptation. A review of published references did not help us either. One possibility is the addition of histidine at the time of glucose adaptation kicks in a nonspecific

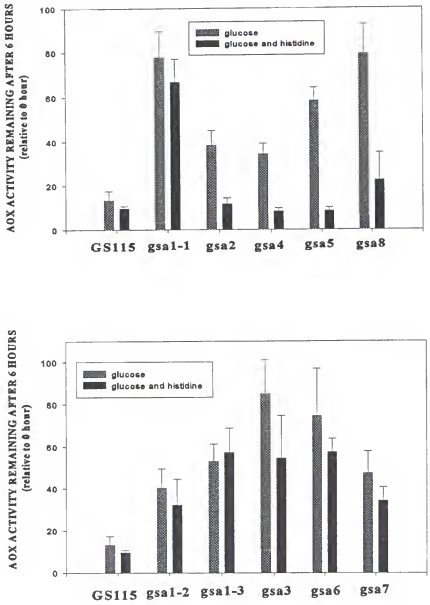


Figure 3-5. AOX degradation and histidine addition during glucose adaptation. The test was used to identify *gsa* mutants that are not affected by histidine addition The experiment was done as a liquid media AOX assay described in Materials and Methods except that at the zero time point of glucose addition, glucose or glucose/histidine were added to each *gsa* mutant to compare the effect of histidine addition. All assays were measured at least three times and the values shown on the graph represent the average ± sd.

protein degradation pathway. It is also possible that histidine may directly activate macroautophagy. To further probe this problem, I first screened all the mutants that I have and test their ability to degrade AOX when histidine was added at the time of glucose adaptation. It was not surprising to find that *gsa1* was not rescued by histidine. Using non-backcrossed *gsa2* strain for the same experiment, I verified that the histidine rescue effect exists and concluded that the phenomenon was not due to improper selection during the backcrossing. All other *gsa* mutants were tested for the effect of histidine addition at the beginning of glucose adaptation and Fig. 3-5 showed the AOX activities remaining after a six-hour glucose adaptation in these *gsa* mutants. The effect of histidine addition was compared with control groups grown in the same condition except no histidine addition at the onset of glucose adaptation. As shown in the upper panel, histidine restored AOX degradation ability (or partially restored) in *gsa2, 4, 5* and *gsa8* while in *gsa1*, *gsa3*, *gsa6* and *gsa7*, histidine has no effect on their AOX degradation. However, the rescue effect varied in these three strains which may be a reflection of the expression level of histidine. *Gsa1*, *gsa3*, *gsa6* and *gsa7* were chosen for further genetic studies.

Morphological Studies of *gsa* Mutants

The ultrastructural analysis was done by the potassium permanganate fixation protocol (Veenhuis, et al., 1983). The basic phenomena in all these

mutants were their inability to degrade peroxisomes in a prompt manner albeit these eight mutants showed distinct presentations (Fig. 3-6 and Fig. 3-7). In *gsa1-1* mutant, the vacuole appeared round and small. It lacked the finger-like extensions or invaginations present in glucose-adapting GS115 cells (see Fig. 1-3). The morphological presentation of the vacuole did not change much during glucose adaptation. The vacuole was not active since there were few autophagosomes and little cell debris inside the vacuole when compared to GS115 cells. All this suggested that *gsa1-1* is required for the initiation of microautophagy and the molecule mutated in *gsa1-1* may control an early step of microautophagy, that is, the initiation of autophagy. The limited degradation of AOX and FDH probably is due to inefficient degradation of peroxisome via macroautophagy under glucose adaptation conditions.

In *gsa4*, the peroxisomes were identified outside of the yeast vacuole after 3 hour's glucose adaptation while autophagosomes were seen associated with the yeast vacuole. Although AOX degradation was defective in *gsa4* cells (see Fig. 3-7), the extent of the defect, when judged by AOX degradation, was much less when compared with other mutants (Fig. 3-3). This was in agreement with the morphological data. We knew in *gsa4*, cytosolic protein FDH's degradation proceeds normally. It is possible that the defect in *gsa4* was mild and although degradation of AOX ability was inhibited, the cytosolic protein FDH degradation still could proceed via impaired microautophagy or inefficient macroautophagy. It is also possible that the mutant has a recognition defect

and this defect disrupted the microautophagy of peroxisomes while its cytosolic protein degradation in *gsa4* cells was intact. It should be interesting to pursue *gsa4* to identify the defective gene of this mutant.

The genetics data showed that *gsa8* has a dominant trait. When examined under electron microscopy, it bore similarity to *gsa4* cells albeit to less extent than to the active level of the yeast vacuole. During glucose adaptation, autophagosomes with cell debris or cytosolic contents were seen around and inside the yeast vacuole (Fig. 3-6). However, large peroxisomes outside of the yeast vacuole were easily identified, and we did not observe vacuolar degradation of peroxisomes. Our biochemical data showed that the AOX degradation was defective. However, FDH degradation proceeded normally. I suspected that the slow progress of macroautophagy leads to a much greater loss of cytosolic protein FDH while the defect in microautophagy keeps AOX level relatively higher in *gsa8*. *Gsa4* and *gsa8* should be in the same group and their defective gene products control the recognition step in the process of peroxisome microautophagy downstream of Gsa1p.

In *gsa2* cells, finger-like extensions of the vacuole were found surrounding the peroxisomes after 3 hour glucose adaptation and this was not seen at the time of methanol induction. However, it appeared that fusion of vacuolar membrane did not occur, and thus, sequestration of peroxisomes was not complete (Tuttle and Dunn, 1995). Although the morphology suggested that microautophagy was shut down in *gsa2* cells, we did observe some small

autophagosomes were in contact with the yeast vacuole. The biochemical data showing that AOX degradation was defective corroborated this observation. However, slow degradation of AOX and FDH did exist and it appeared that the *gsa2* cells might be able to adapt to their deficiency and the environmental situation by turning on the macroautophagy pathway albeit much less efficiently. I suspected a fusion step of vacuolar membrane during the engulfment of peroxisomes is defective in *gsa2* mutants and Gsa2p should act downstream of Gsa1p.

Morphologically, *gsa3*, *gsa6* and *gsa7* showed similar characteristics as *gsa2*. The initiation of autophagy was turned on during the glucose adaptation. The vacuole has formed protrusions that surround peroxisomes. However, we did not observe peroxisome degradation in the yeast vacuole. The biochemical data of AOX and FDH activities during glucose adaptation also supported that peroxisome degradation was impaired in all these three mutants. It appears these mutants are defective in an event at or before the fusion of protrusions of vacuolar membrane, a homotypic vacuolar membrane fusion event. I speculated the molecules that are defective in these mutants may either coordinate the fusion steps in a complex or interact in a step by step manner. The proteins may also reside at different compartments such as peroxisomal and vacuolar membrane or in cytosol. In conclusion, *gsa2, gsa3, gsa6* and *gsa7* are classified in a mutant group with defects in homotypic vacuolar membrane fusion.

In *gsa5* cells, during methanol induction, we observed peroxisomes, mitochondrial and cell debris were wrapped with several high density layers of membrane, a hallmark of macroautophagy. This means that macroautophagy has been turned on in this particular mutant during methanol induction, which is unusual. Some of these autophagosomes were in contact with the yeast vacuole. When the cells were examined at 3 hour of glucose adaptation, we observed the same phenomena as in methanol induction. The macroautophagy was apparently still functioning since multiple membrane layers of cell contents could be easily identified. The yeast vacuole has formed membranous protrusions and the sequestration of peroxisomes by vacuolar membrane protrusions was initiated. However, a subsequent step was defective since no peroxisome degradation was observed. The biochemical data showed that AOX and FDH degradation was defective in *gsa5* and supported our conclusion that *gsa5* was a very distinct mutant defective in microautophagy. I speculated that the defective gene product may inversely control a common event of microautophagy and macroautophagy. Defects in this gene product turned on macroautophagy and interfered with microautophagy process during glucose adaptation. However, this is only a simplified explanation. Although I am aware of this mutants is unique in its phenotypic presentation, I put *gsa5* in the same group of vacuolar membrane fusion defect mutants. Further genetic studies of this mutant will reveal the function as well the role of the defective gene product that control the microautophagy and macroautophagy process.

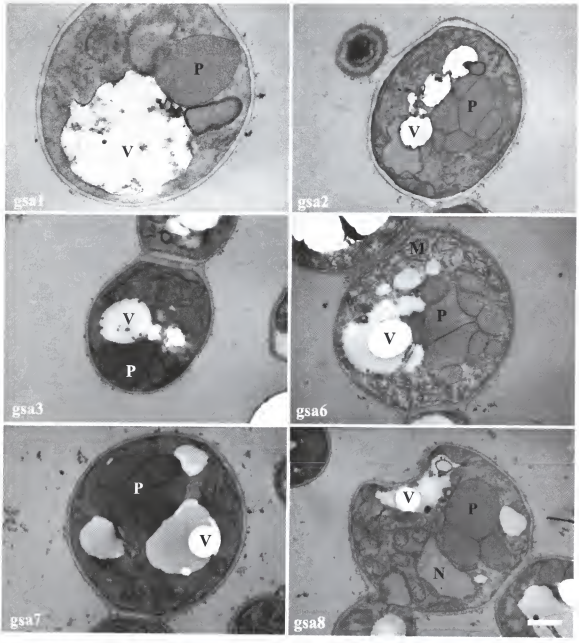


Figure 3-6. Ultrastructural studies of *gsa1-1, gsa2, gsa3, gsa6, gsa7* and *gsa8* mutants under glucose adaptation. *Gsa* mutants were grown on methanol medium and then adapted to glucose for 3 hours. Cells were prepared by a potassium permanganate protocol for ultrastructural analysis (Tuttle and Dunn, 1995). The representative morphology of mutants at 3 hour adaptation is shown here. The detailed description is in the text. N, nucleus; P, peroxisome; V, vacuole and M, mitochondria. Bar: 0.9 μM.

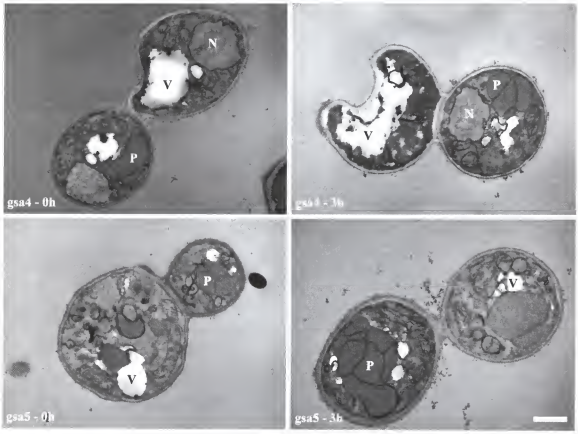


Figure 3-7. Ultrastructural studies of *gsa4* and *gsa5* mutants under glucose adaptation. *Gsa4* and *gsa5* cells were grown on methanol and then adapted to glucose for 3 hours. Cells of 0h and 3h adaptation were prepared by a potassium permanganate protocol for ultrastructural analysis (Tuttle and Dunn, 1995). The representative morphology of *gsa4* and *gsa5* is shown here. The detailed description is in the text. N, nucleus; P, peroxisome; V, vacuole and M, mitochondria. Bar: 0.7μM.

In conclusion, The morphological and biochemical characteristics in all eight *gsa* mutants clearly suggested that the gene products defective in these *gsa* mutants regulate different steps in peroxisome microautophagy. Further genetic studies will reveal their identities and help us to understand the mechanism of autophagy.

Chapter Summary

In this chapter, I have characterized most of the available mutants in our stocks and eight *gsa* complementation groups (*gsa1* to *gsa8*) have been identified. Strain *gsa1-1*, 2, 3, 5, 7, and *gsa8* have been backcrossed two to four times and are ready for further genetic studies. *Gsa1-1* to *gsa8* have also been characterized biochemically and morphologically. All *gsa* mutants lacked the ability to degrade peroxisomes during glucose adaptation. However, their abilities to degrade peroxisome during ethanol adaptation were not affected. I have divided these mutants into four groups. The first group includes *gsa1*, which is defective in the initiation of microautophagy. The second group includes *gsa4* and *gsa8* that have selective degradation defect in AOX but not FDH in peroxisome autophagy. A recognition step might be defective in these two mutants. The third group includes *gsa2, gsa3, gsa6* and *gsa7*. Our data suggested that they are homotypic vacuolar membrane fusion mutants

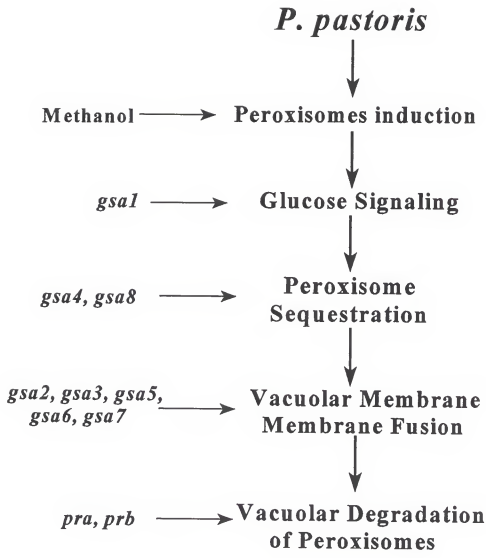


Figure 3-8. Microautophagy of peroxisomes and *gsa* mutants in *P. pastoris*. The process of microautophagy of peroxisomes is depicted here based on the biochemical and morphological evidence we had (see text). Mutations in specific *GSA* genes acted at the specific stages of microautophagy are also indicated. All GSA genes seems act at a step before vacuolar degradation. The vacuolar degradation mutants *pra* and *prb* are also shown in the diagram to complete the microautophagy pathway.

downstream of Gsa1p. The final group includes *gsa5*. In this group, macroautophagy is abnormally turned on under both methanol and glucose growth condition while a fusion step of vacuolar membrane protrusions of microautophagy is shut down as in group 3. As shown in the diagram, all mutants are defective at a step before vacuolar degradation. The representative vacuolar protein degradation mutant *pra* and *prb* are also included in the diagram. Although all defects in *gsa* mutants are specific for glucose-induced microautophagy, the other vacuolar degradation pathway(s) might share a common degradation step or, a common regulatory molecules with the microautophagy pathway. An example of this is microautophagy and macroautophagy at least share a common step of vacuolar degradation and in *gsa5*, the mutated gene product regulates a step in microautophagy buy might also control a step in macroautophagy. The distinct morphological and biochemical phenotypes described in this chapter hint that the defective genes in these *gsa* mutants may regulate different steps of microautophagy of peroxisomes (Fig. 3-8). The next step after mutant characterization is to identify the defective genes in the mutants and to study the functions of their gene products in the microautophagy process. Some of the mutants in our original possession such as WDY9, 11, 12, 14 and WDY15 showed variable biochemical results and thus we chose not to study these mutants further at this time. *Gsa6* cannot be backcrossed to arginine auxotrophs. However, since it is a stable mutant,

we can rescue it without backcrossing. *Gsa8* has a dominant trait and thus classic rescue study is not applicable for it. A clone bank could be made from this strain and the gene can be identified by screens for autophagy defects after transformation. In the following chapters, I will discuss the genetic studies conducted in *gsa1-1* and *gsa7* mutants that we believed control the steps of initiation and vacuolar membrane fusion events of microautophagy of peroxisome.

CHAPTER 4
GSA1 PROTEIN IS PFK1 PROTEIN

Introduction

Our studies presented in chapter 3 showed WDY2 *(gsa1-1)* is a genetically stable and a morphologically distinct mutant. It probably signals the initiation of peroxisome microautophagy. We chose it as the first mutant for further genetic studies. As mentioned in the chapter 3, we followed the experimental steps (see Fig. 3-1) by first backcrossing WDY2 four times. This eliminated the multiple mutations generated during chemical mutagenesis that were not related to *gsa1-1* phenotype. We then complemented *gsa1-1* with a genomic DNA library to recover the gene that corrected the defect in *gsa1-1*. In the following two chapters, I will detail my pursuit of Gsa1p.

Gsa1-1 Signaling an Upstream Event of Microautophagy

Microautophagy of Peroxisomes is Defective in *gsa1-1*

As mentioned in chapter 2, I used peroxisome alcohol oxidase (AOX) and cytosolic formate dehydrogenase (FDH) as a quantitative index to monitor

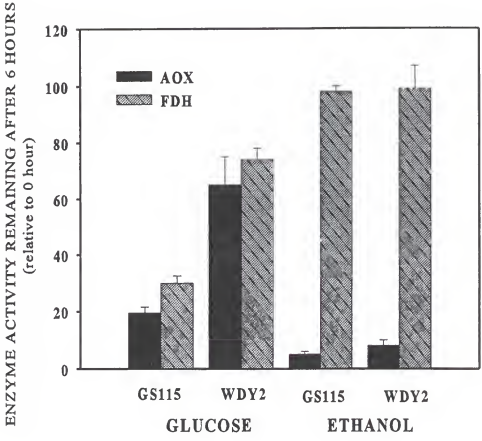


Figure 4-1 Glucose and ethanol adaptation in parental GS115 and WDY2 *(gsa1-1)* mutant. The production of peroxisomes was induced first by culturing the cells in methanol induction medium. After 24-36 hours, glucose or ethanol was added to the medium at final concentrations of 2% and 0.5%, respectively. Cell free extracts were prepared at 0 and 6 hours of adaptation. Alcohol oxidase (AOX) and formate dehydrogenase (FDH) activities were assayed as described in Materials and Methods and presented as a percentage of the activity measured at 0 hour. The values represent the mean ± s.d. of three or more determinations.

peroxisome microautophagy of *gsa1-1* and GS115 during glucose adaptation. As shown in Fig. 4-1, the glucose-induced degradation of both AOX and FDH was dramatically reduced in *gsa1-1* when compared with that of parental GS115. Meanwhile, the loss of AOX in *gsa1-1* cells during six hours of ethanol adaptation was comparable to that observed in GS115 cells. FDH degradation was not observed under ethanol adaptation as in GS115. The data suggested that the mutation in *gsa1-1* affected only the glucose-mediated degradation pathway, while its ethanol-mediated degradation pathway proceeded normally. In addition, vacuolar function appeared normal in the *gsa1-1* cells since under the ethanol-mediated degradation pathway, the vacuolar degradation of peroxisome is intact. Our previous studies have shown that both degradation pathways shared a common vacuolar degradation step. In conclusion, our biochemical data supported that *gsa1-1* is a glucose-mediated peroxisome autophagy mutant with an early event defect.

Morphological Study Revealed That a Step Before Peroxisome Sequestration Is Blocked in *gsa1-1*

We next examined the ultrastructure of *gsa1-1* cells during glucose adaptation. Large peroxisomes were induced and the yeast vacuole was small and round in parental GS115 cells at zero hour of adaptation. When GS115 cells were adapted to glucose, the peroxisomes were few in number when compared with 0 hour glucose adaptation. They were found to be associated with the vacuole. The vacuole contained cellular structures such as

peroxisomes and cell debris (Fig. 1-3). In addition, finger-like extensions of the vacuole surrounding peroxisomes were common. In *gsa1-1* cells at 0 hour of adaptation, *gsa1-1* cells had large peroxisomes that have been induced by methanol while the yeast vacuole was small and round. After three hour of glucose adaption, the peroxisomes were found outside the vacuole in *gsa1-1*. The vacuole appeared "inactive", that is, it lacked cellular contents, membrane invaginations, and finger-like extensions. Also shown in Fig. 4-2 is the WDY1 (*gsa2*) mutant. When comparing the morphology of these two mutants, it supported the hypothesis that *gsa2* is different from *gsa1-1* and Gsa1p should act before Gsa2p.

The ultrastructural data and the biochemical data of *gsa1-1* and *gsa2* suggested that both gene products *GSA1* and *GSA2* are required for the delivery of peroxisomes to the vacuole for degradation during glucose adaptation. I suggested that microautophagy was mainly shut down in both mutants in early steps of microautophagy and that the limited autophagy observed in our biochemical analysis occurred by inefficient macroautophagy under glucose adaptation condition. The different morphological presentations of *gsa1-1* and *gsa2* showed that their gene products control different events of peroxisome microautophagy. My observations supported the conclusion that Gsa1p controls the initiation of microautophagy while Gsa2p controls a downstream event of microautophagy after Gsa1p such as the fusion step of vacuolar membrane during the engulfment of peroxisomes by yeast vacuole.

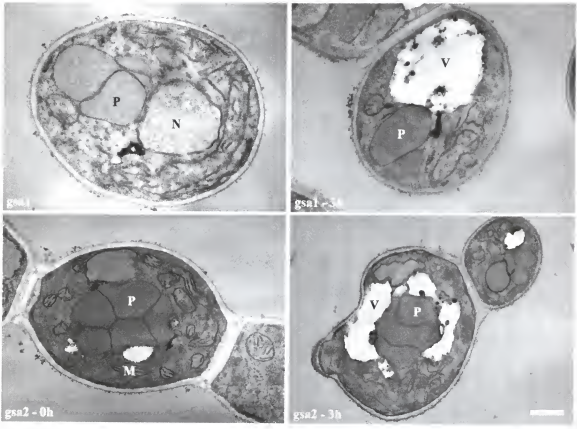


Figure 4-2. Morphology of *gsa1-1* and *gsa2* under glucose adaptation at 0h and 3h time points. The ultrastructural analysis of *gsa1-1* and *gsa2* was done by a potassium permanganate fixation protocol of yeast cells at 0h and 3h of glucose adaptation. At 3h of glucose adaptation, *gsa1-1* showed no morphological change when compared to 0h. However, in *gsa2*, yeast vacuole formed protrusions to wrap the peroxisomes. This is an indication that the defective gene in *gsa2* probably controls a step downstream of *gsa1-1* in the microautophagy process. M: mitochondrion; N: nucleus; P: peroxisome; V: vacuole. Bar: 0.9 μM.

pDLT1 Complemented *gsa1-1* Phenotype

The biochemical and morphological studies have suggested that Gsa1p control the initiation step in microautophagy. I chose *gsa1-1* for further genetic analysis. The main purpose of genetic study is to recover the gene that is defective in a specific *gsa* mutant. A *P. pastoris* genomic DNA library (a generous gift of Dr. J. M. Cregg, Oregon Graduate Institute) was made and put into an episomal shuttle vector pYM8. WDY2 (*his4*, *gsa1-1*) was transformed with the genomic library and plated on transformation medium lacking histidine. The resulting transformants were screened by an AOX direct colony assay to test their ability to degrade AOX in *gsa1-1* cells (Tuttle and Dunn, 1995). If the cell acquired the *GSA1* gene and the gene were transcribed, it should rescue the defect of *gsa1-1* and thus regain the ability to degrade AOX. When examined on nitrocellulose by direct colony assay, the rescued colonies should appear white while non-rescued should appear purple. Of the fourteen putative rescued colonies that were isolated by Dr. Dan Tuttle, only two maintained the wild type ability to degrade AOX when assayed in liquid media. The plasmid DNA isolated from these two clones was amplified in *E. coli* DH5α cells. Both vectors contained a 5.7 kb genomic DNA insert that displayed identical restriction maps. I next tried to verify the ability of this plasmid, pDLT1, to complement the mutant *gsa1-1* cells by reintroducing it back to *gsa1-1*. A 3.8 kb BamH I fragment was also subcloned into pYM8 (pDLT-BB) and used to

transform *gsa1-1*. Since the instability of the episomal vector, pYM8, contributed to inconsistent measurements of AOX degradation, we induced stable integration of these constructs prior to measuring their ability to complement the *gsa1-1* phenotype through YPD-YND-YPD non-selection and selection media growth procedure. Those *gsa1-1* cells stably integrated with pDLT1 and pDLT-BB efficiently degraded AOX during glucose adaptation (Fig. 4-3 and 4-4) in a manner comparable to GS115. This result showed that 5.7 kb insert containing pDLT1 could restore AOX degradation ability of *gsa1-1* cells.

Rescue Study of Different Fragments in the Insert of pDLT1

We next tried to further define regions of the 5.7 kb DNA insert in pDLT-1 that was responsible for complementing the defect of *gsa1-1* (Fig. 4-3 and Fig. 4-4). Restriction fragments of the 5.7 kb genomic DNA were subcloned into pYM8 and pYM4 (Fig. 2-1). Using these constructs, stable transformants of *gsa1-1* were isolated and their ability to degrade AOX during glucose adaptation examined. Stable integration of the pDLT1 constructs would presumably occur at the locus homologous to the genomic DNA insert, since pYM8 contained only 164 nucleotides of DNA homologous to *P. pastoris*. A 3.8 kb BamH I fragment (pDLT-BB) rescued the *gsa1-1* phenotype, while the DNA insert without this 3.8 kb fragment (pDLT-ΔB) did not complement the mutation. I also examined whether smaller fragments from within this 3.8 kb region could

rescue *gsa1-1* or not. My results showed that all fragments tested such as the 2.3 kb Hind III / BamH I fragment (pDLT-HB) did not rescue *gsa1-1* and the minimum rescue fragment is 3.8 kb BamH I fragment. Since pDLT-HB and pDLT-XB did not rescue *gsa 1-1*, the critical rescue part probably lies between BamH I(644) to Hind III (2260) sites. Once the smallest rescue DNA fragment was identified, the next step was to sequence this 3.8 kb fragment. The result of sequence is shown in next section.

Sequencing and Sequence Analysis of pDLT1 Insert

The 3.8 kb BamH I fragment was subcloned into pBluescript and sequenced in both directions by nested deletion methods. The sequence revealed one long open reading frame without a start codon and five short open reading frames encoding proteins of 70 - 115 amino acids (Fig. 4-4). The short open reading frames did not match any amino acid sequences in protein databases accessible to NCBI, but the large open reading frame showed close amino acid homology to the α-subunit of phosphofructokinase (Pfk1p) of *S. cerevisiae* and *Kluyveromyces lactis*. We next sequenced the initial 644 nucleotides of the genomic DNA insert of pDLT-1. The start codon of *PFK1* was identified in addition to 161 base pairs of non-coding 5' upstream DNA. No additional open reading frames were revealed.

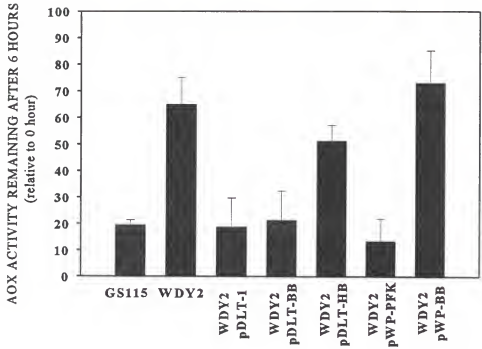


Figure 4-3. pDLT1 rescues the *gsa1-1* phenotype. pDLT1 was recovered by Dr. Dan Tuttle by transforming a genomic DNA library of *P. pastoris* and then screened the rescue colonies that appeared white on the direct colony assay. The recovered plasmid was identified as pDLT1 and it rescued *gsa1-1* by reintroducing it into *gsa1-1* cells. A 3.8kb BamH I fragment cut from pDLT1's 5.7 kb insert also rescued the mutant phenotype in vector pYM8 (pDLT-BB) but not pYM4 (pWP-BB). The rescue result of a full length *PFK1* gene (pWP-PFK) is also shown here. A Hind III - BamH I fragment (see next figure) did not rescue *gsa1-1*. AOX activities were tested in a six-hour glucose adaptation. The values of AOX activities were represented as a percentage of 0 hour values. All assays were measured at least three times and the values shown on the graph represent the average ± sd.

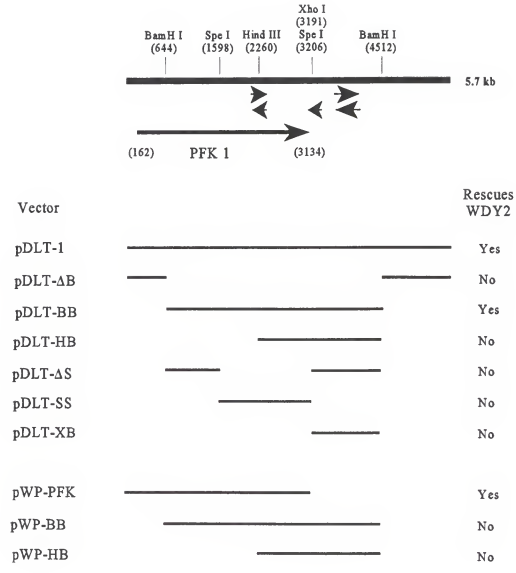


Figure 4-4. Identification of the genomic DNA that rescues *gsa1-1* during glucose adaptation. Genomic DNA fragments were subcloned into pYM8 (pDLT) or pYM4 (pWP) vectors and used to stably transform *gsa1-1*. GS115, *gsa1-1* and transformed *gsa1-1* cells were grown on methanol for 24-36 hours then adapted to 2% glucose medium. Cell free extracts were prepared at 0 and 6 hours of adaptation and assayed for alcohol oxidase (AOX) activities. Glucose-induce degradation of AOX in those *gsa1-1* cells that had been rescued by complementing DNA was rapid and comparable to parental GS115 cells (e.g., pDLT-BB). However, when *gsa1-1* cells were transformed with non-complementing DNA, AOX degradation was slow and comparable to non-transformed *gsa1-1* mutants (e.g., pDLT-HB).

The smallest complementing DNA had five short putative open reading frames with start codons. Three of these open reading frames resided within pDLT-HB while two resided in pDLT-SS. Neither construct was capable of rescuing *gsa1-1* (Fig. 4-3). Since we were stably integrating the genomic DNA prior to testing its ability to complement *gsa1-1*, the smallest complementing DNA does not necessarily represent the entire open reading frame of the rescue gene in *gsa1-1*. Indeed, we suggest that the 3.8 kb BamH I fragment complemented *gsa1-1* by correcting the mutation upon homologous recombination into the *PFK1* locus.

In a parallel experiment, I also integrated the complementing DNA fragments into the *HIS4* locus of *gsa1-1* and tested their ability to rescue *gsa1-1*. This was done by constructing the 2.3 kb Hind III / BamH I fragment, the 3.8 kb BamH I fragment and the complete *PFK1* gene including 161 nucleotides of 5' upstream DNA into pYM4, which contains a *P. pastoris HIS4* gene. Prior to transformation these vectors were linearized by a single cut within the *HIS4* gene to direct their integration to the *HIS4* locus. Under these conditions, neither pWP-HB nor pWP-BB rescued *gsa1-1* (Fig. 4-4). We propose that the inability of pWP-BB to complement was due to the absence of the *PFK1* start codon when the vector integrated into the *HIS4* locus. Indeed, when the start codon was present, *PFK1* (pWP-PFK) complemented the *gsa1-1* phenotype (Fig. 4-4). This experiment showed that PFK is responsible for the rescue effect of *gsa1-1*. Our morphological studies of mutants and their transformants

showed that PFK restored the ability of *gsa1-1* cells to degrade peroxisome (Fig 5-5 and Fig 5-6). The results that Pfk1p might be Gsa1p was a surprise for me since PFKp has identified function in glycolysis. Before I could reach the conclusion that Pfk1p is Gsa1p, I needed more data about *gsa1-1*.

Northern Blot Analysis of *gsa1-1* Mutants

At the time of identifying genomic DNA that rescues *gsa1-1*, I also tried to use Northern analysis to 1) identify possible open reading frames that might transcribe in this 3.8 kb region and 2) observe the message RNA (mRNA) level of *PFK1* in different yeast strains. A 2.5 kb BamH I / Xho I DNA fragment inside the pDLT insert was used as a probe to identify on Northern blots those mRNA's of GS115, WDY1(*gsa2*) and WDY2 (*gsa1-1*) that were being transcribed *in situ*. One predominant mRNA at 3.2 kb and two minor mRNA's at 2.7 and 1.5 kb were identified in GS115 cells (Fig. 4-5). The 3.2 kb mRNA matched the predicted size for *PFK1* mRNA. Neither 2.7 kb nor 1.5 kb mRNA corresponded with any predicted open reading frames within the *PFK1* locus (Fig. 4-4). We believed they bound to the membrane nonspecifically. The 3.2 kb mRNA was also present in *gsa2*, but was greatly diminished in *gsa1-1* cells. These results could not be attributed to sample loading since the amounts of 18S and 28S rRNA loaded onto the lanes were comparable. It is possible that the 3.2 kb mRNA was neither transcribed nor stable in *gsa1-1* cells. A mutation

within the promoter region may suppress transcription, while a premature stop codon would terminate translation causing a destabilization of the mRNA. Our studies suggested that the mutation was not within the upstream 5' region but within the *PFK1* encoding region, since pDLT-BB which lacked bases -161 through +483 of the *PFK1* gene was able to rescue *gsa1-1* when stably integrated (see Fig. 4-4). This experiment showed that *PFK1* is the only transcribing gene in this region and PFK mRNA was greatly reduced in *gsa1-1* cells and the further evidence that *PFK1* gene is related to *gsa1-1* mutation.

Phosphofructokinase Activity Assay in *gsa1*

The absence of the 3.2 kb *PFK1* mRNA in *gsa1-1* led me to measure the phosphofructokinase activities in these cells (see Materials and Methods). We found that WDY2 (*gsa1-1*) cells had less than 15% of the phosphofructokinase activity present in parental GS115 cells whereas WDY1 (*gsa2*) cells had comparable PFK activity level to that of GS115 (Fig. 4-6). The data were consistent with *GSA1* encodes Pfk1p and *gsa1-1* is a *pfk1* mutant.

Phosphofructokinase is a heterooctamer composed of four α-subunits encoded by *PFK1* and four β-subunits encoded by *PFK2* in yeast cells (Berger and Evans, 1992, Arvanitidis and Heinisch, 1994). The *P. pastoris PFK1* gene is 2.97 kb long encoding a protein of 989 amino acids with a predicted molecular mass of 108.7 kDa (Fig. 4-7). The PFK protein catalyzes the

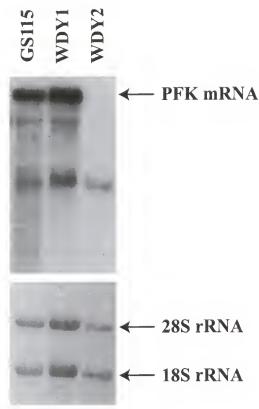


Figure 4-5. Northern blot analyses of GS115, WDY1 (*gsa2*), and WDY2 (*gsa1-1*) cells. Cells were grown in YPD medium, harvested and total RNA prepared. The RNA was separated on an agarose gel and transferred to a nylon membrane. After staining with methylene blue to identify rRNA (lower panel), the membrane was probed with a ^{32}P-labeled 2.5 kb BamH I / Xho I fragment from the original 5.7 kb genomic DNA (see Fig. 4-4). This fragment spans 85% of the *PFK1* open reading frame.

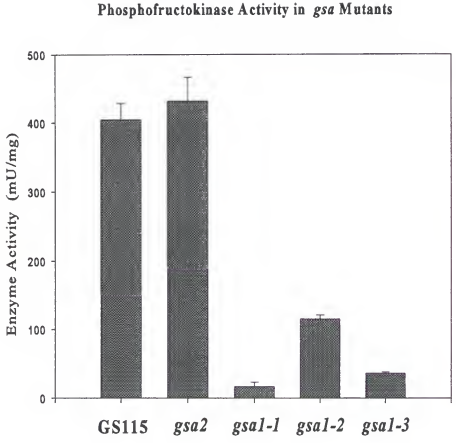


Figure 4-6. PFK activity is greatly reduced in WDY2 (*gsa1-1*). Since the sequence data and Northern blot showed that *PFK1* might be *GSA1*, we measured the activity of PFK protein. The parental GS115 and *gsa2* showed normal PFK activity while the activity of PFK protein in *gsa1-1* reduced greatly (less than 10%). This clearly showed that we recover the right gene. The PFK activities of *gsa1-2* and *gsa1-3* are also included. Interestingly, the activity levels in these three *gsa1* mutants are totally different. All assays are measured at least three times and the values shown in the graph represent the average ± sd.

conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. The enzyme controls one of the rate limiting steps in glycolysis and is highly regulated by many metabolites including fructose 2,6-bisphosphate, AMP and citrate. The catalytic and regulatory sites of the Pfk1 protein are highly conserved between *P. pastoris*, *S. cerevisiae* and *H. sapiens* (Fig. 4-8). For example, there exists 60-85% amino acid identity within the fructose 6-phosphate binding site, the ATP binding site, the fructose 2,6-bisphosphate binding site, the AMP binding site, and the citrate binding site (Arvanitidis and Heinisch, 1994). Interestingly WDY3 (*gsa1-2*) and WDY8 (*gsa1-3*) previously identified as the same *gsa* group of WDY2 (*gsa1-1*) also showed decrease of PFK activity. This also verified that they belong to the same *gsa1* complementation group. However, *gsa1-2* showed only a two-thirds decrease of the activity when compared with GS115 cells and in *gsa1-3*, about 20% activity remained. I speculate that different activity levels of PFK reflected different missense or nonsense mutation sites in these mutants. For example, in *gsa1-2* and *gsa1-3*, the mutation may well be missense but at important positions such as a substrate binding site or an ATP binding sites. If PFK protein in *P. pastoris* is also composed of α- and β-subunits as in *S. cerevisiae*, the mutations in *gsa1-2* and *gsa1-3* could also be in *PFK2* gene. These strains are very useful for future studies. Based on the ability of genomic DNA fragments to rescue the *gsa1-1* cells and the absence of *PFK1* mRNA, we speculate that the *gsa1-1* mutant has a premature stop codon near the N-terminus of the *PFK1* gene. This would

result in the synthesis of a truncated mRNA which may lead to the instability of *PFK1* mRNA as we observed on our PFK mRNA study. The translation could also be prematurely stopped.

Carbohydrate metabolism is complicated in yeast and some of the pathways are still not fully understood because different yeast strains show different pathways and abilities to metabolize sugars (Wills, 1990, Wills, 1996). However, evidence has accumulated that *S. cerevisiae* can grow on glucose in *pfk* mutants (Lobo and Maitra, 1983, Schmitt, et al, 1984). Glucose is metabolized via the pentose phosphate pathway in *pfk* mutants (Schmitt, et al, 1984, Heinisch and Zimmermann, 1985, Jacoby et al, 1993, Boles, et al, 1993). This pathway then joins the glycolysis at glyceraldehyde-3-phosphate level (Wills, 1990). This may also be the case in *P. pastoris*. Sibirny et al. (1987) found that when *Pichia pinus* cells were grown on methanol, the enzymes for methanol utilization were greatly induced. The subsequent glucose induced catabolite repression of transcription of methanol metabolism enzymes is controlled by the glucose catabolite repression gene 1 (*GCR1*). By using glycolysis enzyme assay, they found that the *gcr1* mutant has low level of PFK activity. They have evidence that in *gcr1* mutant, the loss of repression was not due to the damage of the glycolysis pathway. However, no follow-up studies of this *gcr1* mutant can be found. This was an indirect evidence that Pfk1p might participate in glucose repression of AOX protein via autophagy.

```
-161  GGATCTTTCTTCTCTTGCTATAAATCAAACATTATTCATACAGAGTTTAATCGA
-107  TTCAACAAAGCATAACATTGATTGCAATTGGTTCCGTCTTGAACTGCTAAGGAG
 -53  AACAATCATAAATTAGTATTTTGTTTGCTTGTTAGACTCAAATCGAATTACAGA
   1                                                       M
   2  TGCCAGAACCATCTATAAGTGACTTTCCTTCACTTCGTTTGTCACTAATGATG
   2  P  E  P  S  I  A  S  A  L  F  S  T  S  F  V  T  N  D  D
  56  ACAAACTGTTTGAAGAGACTTTCAATTTTTACACGAAGTTGGGCTTCCACGCAA
  20  K  L  F  E  E  T  F  N  F  Y  T  K  L  G  F  H  A  T
 110  CACGCTCATATGTTAAAGACAACCGGTCAGACTTTGAATTGACGGGGATTTCCA
  38  R  S  Y  V  K  D  N  R  S  D  F  E  L  T  G  I  S  T
 164  CGGATTCAATCAAGGAAATCTGGCTGGAAAGTTTCCCACTATCTGAAGTGGTCG
  56  D  S  I  K  E  I  W  L  E  S  F  P  L  S  E  V  V  E
 218  AAACGTCAGCTGGTAGAGAGTTGAGAAAACCACTGCAAGAATCTGTGGGCTACC
  74  T  S  A  G  R  E  L  R  K  P  L  Q  E  S  V  G  Y  Q
 272  AATCTGAAGCTTCTTCTGGAATATTCTCCCTACCAGAGTGACGGTGTTGTTATAA
  92  S  E  A  L  L  G  Y  S  P  Y  Q  S  D  G  V  V  I  K
 326  AATTAAGGTTATCAAATCATGACCTTCGAAAAACAAAGACTTGCCCGGTGAAG
 110  L  R  L  S  N  H  D  L  K  Q  K  N  K  D  L  P  G  E  V
 380  TTACGTTTTCCACCGCTAGTATCGACAAATTAAGAGCTAAACTCATTGAAATTG
 128  T  F  F  T  A  S  I  D  K  L  R  A  K  L  I  E  I  G
 434  GTGCTGAGATAATTCCCTCAGAAATAGACCTTGTTGAATTTTCAACCAAGGATC
 146  A  E  I  I  P  S  E  I  D  L  V  E  F  S  T  K  D  P
 488  CTATGGGCGACGTCATTAGCTTTTCTTCTTATCCCTCTTTGAGTTCCAAGAAGA
 164  M  G  D  V  I  S  F  S  S  Y  P  S  L  S  S  K  K  E
 542  AGATTACCTCTCCAGACTTTTCCTCCCTACCAAGAAGGAAGTACGCTCCCAAG
 182  I  T  S  P  D  F  F  L  H  P  K  K  E  V  R  S  D  E
 596  AATCAATAGTTGAGCAGGTTAAATCTGAAGAAGGTAAGAAGAAGATTGCCATCA
 200  N  Q  I  V  E  Q  V  K  S  E  E  G  K  K  K  I  A  I  E
 650  TAACTTCAGGTGGAGACGCACCGGGAATGAATGCTGCAGTAAGGGCTGTGACAA
 218  I  T  G  G  D  A  P  G  M  N  A  A  V  R  A  V  T  R
 704  GAGCCGGTATTTTCTATGGCTGTAAAGTTTACGCTTGTTATGAAGGTTACACTG
 236  A  G  I  F  Y  G  C  K  V  Y  A  C  Y  E  G  Y  T
 758  GACTGGTTAAGGGTGGTGATATGTTAAGGAAACTGCAGTGGCAAGATGTCCGTG
 254  L  V  K  G  G  D  M  L  K  E  L  Q  W  Q  D  V  R  G
 812  GTTTACTTTCCATTGGTGGTACCATAATTGGTACTGCAAGAAGTAAGGAATTCA
 272  L  L  S  I  G  G  T  I  G  T  A  N  S  K  E  F  R
 866  GAGAACGATGGGGCCGTCTTCAAGCTTGCTACAATATGGTCAGCAATGGTATTG
 290  E  R  W  G  R  L  Q  A  C  Y  N  M  V  S  N  G  I  D
 920  ATGCGTTAGTGTTGTTGTGGAGGTGACGGATCTCTTACAGGTGCCGATCTATTTC
 308  A  L  V  V  C  G  D  G  S  L  T  G  A  D  L  F  R
 974  GAAATGAAGTGCCTGAACTGATAAAGGAACTTTTGGGTGAGGCAAAATTACAA
 326  N  M  P  E  I  L  E  L  K  E  L  G  K  A  I  K
1028  AAGAACAATATGAAACACACAGAAACTTGACAATCGTAGGTCTCGTTGGTTCTA
 344  E  D  Y  E  T  H  H  N  T  L  I  V  G  L  V  G  S  I
1082  TCGATAACGATATGTGCGGAACTGATTCCACAATTGGTGCTTATTCCTCATTGG
 362  D  N  D  M  C  G  T  D  S  T  I  G  A  Y  S  S  L  E
1136  AGAGAATCATAGAGCTGGTAGACTACATCGATGCTACTGCCGCCTCCCATTCAC
 380  R  I  E  L  V  D  Y  I  D  A  T  A  A  S  H  S  R
1190  GAGCCTCTCGTGGTGGAAGTCATGGGTAGACATTGTGGATGGTTAGGTTTAATGT
 398  A  F  V  V  E  V  M  G  R  H  C  G  W  L  G  L  M  S
1244  CCGGAATTGCTACTGGAGCTGATTACATTTTCATCCCTGAAAGACCTCAAGTG
 416  G  I  A  T  G  A  D  Y  I  F  I  P  E  R  F  P  S  E
1298  AAACAAACTGGAAGGACGACTTGAAGAAAGTCTGTTTGAGACATAGAGAAAG
 434  T  N  W  K  D  D  L  K  K  V  C  L  R  H  R  E  K  G
1352  GACGCAGGAAGCACCACCGTTATTGTTGCTGAAGGTGCTATTGATGACCAACTGA
 452  R  R  K  T  I  V  I  A  E  G  A  I  D  D  Q  L  N
1406  ACCCTATCACTTCTGAAGAGGTGAAAGATGTACTAGTGGAGATTGGTTTGGACA
 470  P  I  S  E  E  V  K  D  V  L  V  E  I  G  L  D  T
1460  CTCGTATTACCCGTCTAGGACATGTCCAAAGAGGTGGAGCTCCGTGTGCTTTTG
 488  R  I  T  R  L  G  H  V  D  R  G  G  A  P  C  A  F  D
1514  ATAGATTCTTGGCCACTGTTCAAGGTGTTGATGCTGTTAGGGCTGTTTTAGAAA
 506  R  F  L  A  T  V  Q  G  V  D  A  V  R  A  V  L  E  S
1568  GCACCCCAGCAATTCCTTCTCCTGTCATCAGCATTTTGGAGAACAAAATTGTTC
 524  T  P  A  I  P  S  P  V  I  S  I  L  E  N  K  I  V  R
1622  GCCAGCCGTTGGTGGAATCTGTTGCTCAAACAAAGACTGTCAGTGATGCTATCG
 542  D  P  L  V  E  S  V  A  D  T  K  T  V  S  D  A  I  E
1676  AGGCCAAGGATTCGAATAAAGCTTTGAAATTAAGAGACCAAGAGTTTGCCACAT
 560  A  K  D  F  D  K  A  L  K  L  R  D  E  F  A  T  S
1730  CATATGAGAGCTTCCTGCTCCGTTTCCAAGTATGACGATGGATCATATCTAGTAC
 578  Y  E  S  F  L  S  V  S  K  Y  D  D  G  S  Y  L  V
1784  CAGAGAGCTCAAGATTAAATATTGCCATCATCCATGTGGGAGCTCCAACATCTG
 596  E  S  S  R  L  N  I  A  I  I  H  V  G  A  P  T  S  A
1838  CGTTGAATCCTGCCACAAGAGTTGCTACTTTGAACTCGTTGGCAAAAGGACACA
 614  L  N  P  A  T  R  V  A  T  L  N  S  L  A  K  G  H  R
1892  GAGTTTTTGCTATTCGAAACGGATTTGACGAGATTAATTCGCCACGGCCTGTAC
 632  V  F  A  I  R  N  G  F  A  G  L  I  R  H  G  A  Y  R
1946  GAGAGCTCAACTGGATTGATGTTGAGGACTGGCACAACACAGGTGGGTCGGAGA
 650  E  L  N  W  I  D  V  E  D  W  H  N  T  G  G  S  E  I
2000  TTGGCACCAACAGAAGTCTTCCTAGTGATGATAGTGGGCACTGCGGCGTACTACT
 668  G  T  N  R  S  L  P  S  D  D  M  G  T  A  A  Y  Y  T
2054  TCCAGCAATACAAGTTTGATGGTCTTATTATATCGGMGGATTTGAAGCTTTCA
 684  S  N  T  S  F  D  G  L  I  I  G  F  E  A  F  K
2108  CAGCTCTGTACCAGCTGGACGCAGCTCCGGCTCAGCATCCTATCTTCAATATTC
 704  A  L  Y  Q  L  D  A  A  R  A  Q  H  P  I  F  N  I  P
2162  CAATGTGTTGNCTTCCAGCTACTGTTTCTAATAACGTTCCTGGTACCGAGTATT
 722  M  C  X  L  P  A  T  V  S  N  N  V  P  G  T  E  Y  S
2216  CCTTAGGGTCTGACACATGTCTAAACACCTTGTCTGGATACTGTGATGCTGTGA
 740  L  G  S  D  T  C  L  N  L  S  G  Y  C  D  A  V  K
2270  AACAATCTGCTTCTGCTAGTAGAAGAAGAACATTTGTTGGAAGTTCAAGGTG
 758  Q  S  A  S  A  R  R  I  F  V  V  E  V  D  S  G
2324  GATACTCAGGATATCTTGCCAGCTACGCTGGTCTGATCACAGGAGCTTTGGCTG
 776  Y  S  G  Y  L  A  S  Y  A  G  L  I  T  G  A  L  A  V
2378  TTTATACTCCTGAAAACCCAATCAACCTTCAAACAGTGCAGGAAGACATTGAAT
 794  Y  T  P  E  N  P  I  N  L  Q  T  V  Q  E  D  I  E
2432  TGTTGACTCGAACATACGAGGAAGACGATGGTAAGAACAGATCGGGTAAAATCT
 812  L  T  R  T  Y  E  E  D  G  K  S  N  R  S  G  K  I  F
2486  TTATTCATAATGAAAAGGCTTCAAAGGTTTACACCACGGATCTGATTGCTGCTA
 830  I  H  N  E  K  A  S  K  V  Y  T  D  L  I  A  A  I
2540  TCATAGGTGAAGCTGGAAAGGGTACGTTTGAGAGCCGATGCTGCCGTGCCTGGTC
 848  I  G  E  A  G  K  G  T  F  E  S  R  T  A  V  P  G  H
2594  ATGTACAACAGGGTAAATCTCCCTCACTATTGACCGGGTTAATGCCTGCAGAC
 866  V  D  D  G  K  S  P  S  I  D  G  V  N  A  A  D
2648  TGGCTATCAAATGTGTAACTTCATCGAGGAGCCCAATTTCCAGGTGAAACACA
 884  A  I  K  C  N  F  I  E  D  A  N  F  Q  V  K  H  N
2702  ATGCCAATTTGAGCGCCGACGAACGTCATTTGAGATTCTTTTGCGATGACGGAG
 902  A  N  L  S  A  D  E  R  H  L  R  F  F  C  D  D  G  V
2756  TTAAGACATCTGCAGTGAGCGGCAAATCTTCCGTGATAGATGATAACAGTCAG
 920  K  T  S  V  S  G  K  S  F  I  D  D  N  T  S  V
2810  TGGTCATTGGAATCCAAGGTTCCGAGGTTACATTCACTCCTGTAAAACAGCTAT
 938  V  I  G  I  Q  S  E  V  T  F  T  P  V  K  Q  L  W
2864  GGGAGAAGGAAACTCATCATAAGTGGCGAAAGGGTAAGACGTTCATTGGGAGC
 956  E  K  E  T  H  H  K  W  R  G  K  N  V  H  W  E  D
2918  AGTTGACATTGTCTCTGACCTCTTGAGTGGTCGTTTGTCTATTCGTACCACGT
 974  L  N  I  V  S  D  L  L  S  G  R  L  S  I  R  T  *
2972  AAAAGACGGATCAAATCGGTTGTTTGGGTACTAAAGACAATCCATTTTTTTCT
3026  TNCTCTCGAGCTGGATGAAACTAGTGCATGTACGAATCCGCGTGTAATCTACTG
```

Figure 4-7. Nucleotide and predicted amino acid sequences of *P. pastoris PFK1* gene. The 5' upstream region in the original 5.7 kb clone included 161 bases. The Pfk1 protein is predicted to be 989 amino acids with a molecular mass of 108.7 kD. The sequence of *PFK1* has a GenBank accession number of U73376.

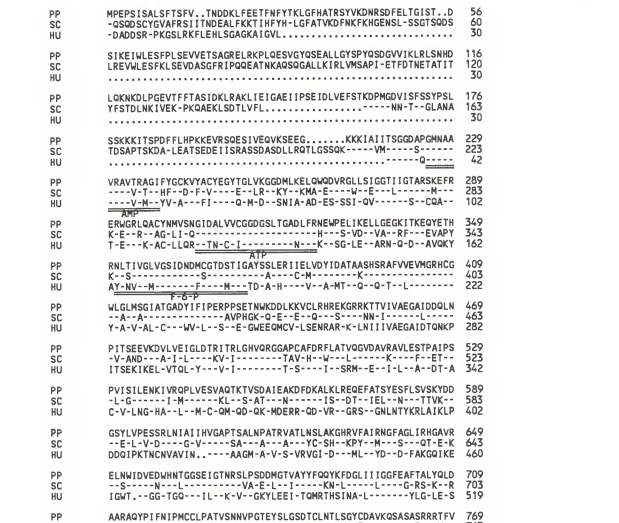


Figure 4-8. Comparison of *PFK1* genes. The sequence of Pfk1p (PP) was aligned to those *PFK1* sequences of *S. cerevisiae* (SC) and *H. sapiens* (HU). Gaps are represented by "dots" (....) and amino acid identity represented by "dashed lines" (-----). Conserved catalytic and regulatory domains sites are also included (Arvanitidis and Heinisch, 1994).

Glycolysis Pathway and *gsa* Mutants

Glucose signals the onset of peroxisome degradation in the glucose adaptation pathway. It may either directly send a signal to initiate the onset of microautophagy of peroxisomal and cytosolic proteins or exert that effect through a subsequent glycolysis enzyme or product. The discovery that *GSA1* is *PFK1* prompted me to probe this question in *gsa1-1* as well other *gsa* mutants. I want to know If glucose is the direct signaling molecule and if other carbon sources could signal the initiation of peroxisome degradation. We asked ourselves which entry point in the glycolysis pathway is related to the autophagy signaling process and if this is related to Pfk1 function in the glycolysis pathway. By providing different carbon sources for yeast to grow, we found that *P. pastoris* could utilize fructose, mannose, glycerol and pyruvate as the sole carbon sources for growth. However, when these carbon sugars were used as adaptation carbon source, we observe pyruvate could not initiate peroxisomal AOX degradation in parental GS115 strain while fructose, mannose and glycerol could do the job as efficiently as glucose in GS115 (Fig 4-9 and Fig 4-10). Pyruvate, fructose and mannose could not initiate microautophagy in *gsa1-1* and *gsa2* mutants while glycerol did initiate the degradation process in GS115 cells as well as in *gsa1* and *gsa2* mutants. In a *PFK1* knockout, a null mutant of *gsa1-1* I isolated later in the experiment also could undergo glycerol induced autophagy (see chapter 5).

These experiment data suggested that 1) Glucose is not the molecule to directly initiate microautophagy since other carbon sources could also do so; 2) A signal was sent out to initiate microautophagy during glycolysis before pyruvate step; 3) The signal might be sent out between glycolysis steps such as fructose-1-phosphate to glyceraldehyde-3-phosphate. Pfk1p is in the middle of these steps and it may well be the initiation signal. However, I speculate that Pfk1p may direct either signal the microautophagy or initiate the microautophagy process through a downstream molecule (not necessarily in the glycolysis pathway). In Fig. 4-10, AOX could be degraded in all mutants when glycerol was used as an adaptation carbon source. It is possible that glycerol addition initiates another yet to be identified peroxisome autophagy pathway. This pathway may share some of the steps that have been defined previously such as vacuolar degradation. In Sibirny's study (1987), they found carbon sources such as glucose, fructose, mannose and galactose repressed the synthesis of alcohol oxidase in the wild type yeast *P. pinas*. The repression with these sugars was impaired in a *gcr1* mutant which was subsequently identified containing a defective Pfk enzyme. However, glycerol repression proceeded normally in this mutant. These findings corroborated our view that glucose is not the direct signal molecule of microautophagy and the carbon repression is related to Pfk protein. However, a downstream enzyme or glycolysis product is still possible to be responsible for the initiation of autophagy process. Further studies are needed to reach a conclusion.

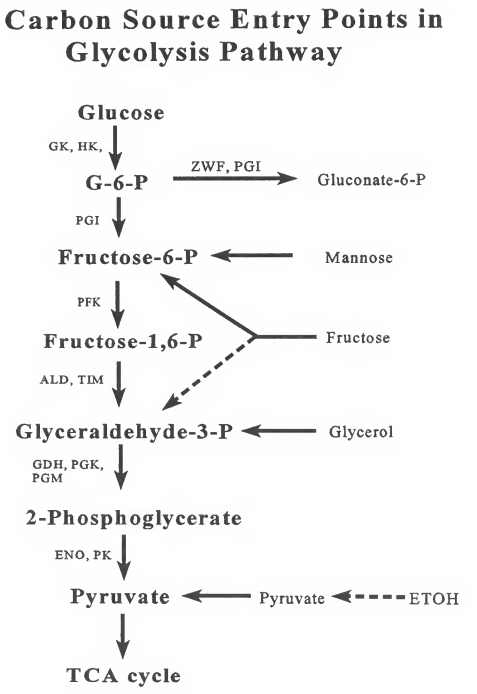


Figure 4-9. Glycolysis pathway and the entry point of carbon metabolites. The flow chart of glycolysis is shown here and the carbon source entry points are also included. The results of induction of peroxisome degradation by these carbon sources are shown in the next figure.

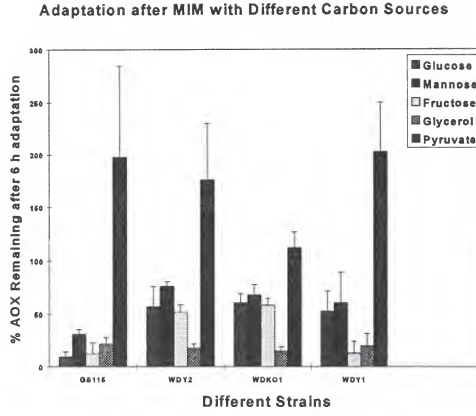


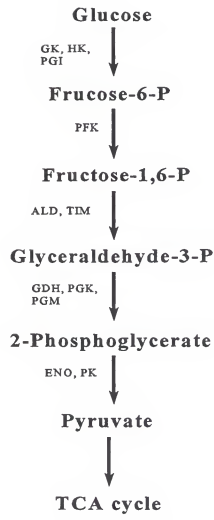
Figure 4-10. Metabolites in the glycolysis pathway and their induction of the degradation of AOX. GS115 cells and mutants were grown on methanol induction media for 24 to 36 hours and then were subjected to adaptation sources such as glucose, glycerol, mannose, fructose and pyruvate. Cell extracts were collected at zero hour and six hours after adaptation. A comparison of AOX activities before and after 6h glucose adaptation was presented here. Values were presented as an average of percentage of the zero hour activity ± sd.

Glycolysis and *gsa* Mutants

Since *GSA1* has been identified as *PFK1*, the question raised by this finding is if other *gsa* mutants are also defective in PFK or other glycolysis enzymes. To approach this questions, I screened all my existing mutants with a PFK activity assay. WDY3 which has been previously identified as *gsa1-2* showed a 70% decrease of its PFK activity (see Fig. 4-6). Another mutant WDY8 also showed 80% decrease of its PFK activity. All other mutants had comparable PFK activity level as parental GS115.

Next I tested the activities of some other glycolysis enzymes in some of the existing *gsa* mutants (see Materials and Methods). The assay included other two main regulatory step enzymes, hexose kinase and pyruvate kinase. The results are shown in Fig. 4-11. When these mutants were examined with available enzyme assays, except for *gsa1-1*, no significant difference was seen in these enzyme activity levels when compared with parental GS115. In *gsa1-1* mutant, PFK activity was greatly reduced by 90% as expected. However, the activity of phosphoglycerate mutase (PGM) of *gsa1-1* increased three fold when compared with parental GS115 and other mutants. The pentose shunt pathway has been identified as an alternative pathway for glycolysis to continue in *pfk* mutants (Heinisch and Zimmermann, 1985, Jacoby, et al., 1993). The cells circumvented the PFK action step in the glycolysis pathway via the pentose shunt pathway to enter glycolysis via glyceraldehyde-3-

Glycolysis Enzyme Assay of gsa Mutants



	GS115	gsa1	gsa5	gsa6	gsa7	gsa8
GK*	387	358	307	358	261	282
HK	153	126	149	119	160	141
PGI	1440	1679	1433	1666	1174	1134
PFK	404	16.5	433	434	421	351
ALD	602	1204	853	942	947	735
TIM	20	16	11	14	18	16
PGM	1129	4420	1175	1238	920	735
ENO	116	99	67	113	49	75
PK	645	593	417	479	517	489

* All enzyme assay values are represented as mU/mg

Figure 4-11 Glycolysis enzyme activities and *gsa* mutants. Glycolysis enzyme assays were applied to test possible glycolysis mutants in our possession. The principle of assays is based on a coupled enzyme assay resulting in the oxidation or reduction of NADH/NAD or NADPH/NADP (see Materials and Methods). The enzymes tested are GK: glucokinase; HK: hexokinase; PGI: phosphoglucose isomerase; PFK: phosphofructokinase; ALD: aldolase; TIM: Triosphosphate isomerase; PGM: phosphoglycerate mutase; ENO: enolase; PK: pyruvate kinase. The values were an average of three measurements.

phosphate. This happens two steps upstream of PGM where it acts. Since glycolysis is altered in *gsa1-1*, I suspected that the change of a particular glycolysis product concentration level may send an up-regulation signal for the transcription of PGM. *Gsa1-1* has been backcrossed and it is unlikely that a mutation in PGM gene promoter region up-regulates the expression of PGM. PGM has been found to undergo rapid dephosphorylation during trichocyst exocytosis in *Paramecium tetraurelia* (Treptau et al.,1995). A protein phosphatase/kinase system was suspected to be involved in the modification of PGM and thus the regulation of exocytosis in *P. teraurelia* cells (Kissmehl, et al., 1997). However, the role of PGM in this exocytosis is still under investigation and debate. Nevertheless, it is surely worth investigating the role of PGM in the autophagy process. The study of glycolysis enzyme assays in these *gsa* mutants showed clearly that the tested *gsa* mutants except for *gsa1* did not involve the tested glycolysis steps. Future genetic studies of the remaining *gsa* mutants will yield useful information about the glucose induced microautophagy.

Chapter Summary

WDY2 *(gsa1-1)* strain was used for the biochemical, morphological as well as genetic studies. My result showed *gsa1-1* is defective in peroxisome degradation during glucose adaptation after methanol induction. Its

peroxisome degradation proceeds normally during ethanol adaptation. The morphological evidence supported that *gsa1-1* is defective in the initiation of microautophagy of peroxisomes. By complementing *gsa1-1* with a genomic DNA library, we were able to recover the plasmid pDLT1 that rescued the *gsa1-1* mutant phenotype. The minimum fragment that rescued *gsa1-1* is a 3.8kb BamH I fragment (BB) within pDLT1. The reintroduction of pDLT1 and pDLT-BB into *gsa1-1* confirmed its rescue effect. The subsequent sequencing showed that the main open reading frame codes the *PFK1* gene. Phosphofructokinase activities as well as *PFK1* mRNA levels were greatly reduced in *gsa1-1* cells. This verified that our cloning of pDLT1 is correct and *gsa1-1* is *pfk1*. However, we knew that Pfk1p is a glycolysis pathway enzyme with defined function. It is a heterooctameric protein composed of 4 α-subunits and 4 β-subunits, a key regulatory enzyme in the glycolytic pathway. The glycolysis enzyme assays of mutants showed other *gsa* mutants are not directly linked to glycolysis. The carbon source adaptation experiments I finished showed the microautophagy signal is sent out between fructose-1-phosphate and glyceraldehyde-3-phosphate. In order to better define the role of Pfk1p in the peroxisome microautophagy process, I need to test if Pfk1p is necessary for the initiation of microautophagy upon glucose addition. I also need to test if the effect of Pfk1p is a direct one or an indirect effect by simply restricting the conversion of fructose-1-phosphate to fructose-1,6-bisphosphate or by up or down regulating other molecules that are specific to peroxisome

microautophagy. If Pfk1p has a direct role in microautophagy, I also need to identify what the functional role of Pfk1p is in peroxisome autophagy. To approach these questions, I first tested if *PFK1* is necessary for the peroxisome autophagy by generating a PFK1 knockout strain *Δpfk1* and comparing its phenotype with that of *gsa1-1*. I also examined the function of Pfk1p in glycolysis and its relationship to peroxisome microautophagy. I will present the results of these studies in the following chapter.

CHAPTER 5
PFK1P IS REQUIRED FOR THE INITIATION OF PEROXISOME MICROAUTOPHAGY

Introduction

In the previous chapter, I have shown that Pfk1p is Gsa1p. However, the question remained unanswered as to what the role of Pfk1p is in the peroxisome microautophagy. It is possible that Pfk1p simply assists in the conversion of glucose to a downstream true effector in glycolysis. It is also possible that PFK protein directly regulates the microautophagy of peroxisomes. Our previous results left these two possibilities still open. As a first step to probe this problem, I tried to verify that the *PFK1* gene is essential for the microautophagy of peroxisomes. I used gene disruption method to knockout the *PFK1* in *Pichia pastoris* and to compare its biochemical and morphologic profiles with that of *gsa1-1*. Then I mutated the catalytic site of the Pfk1 protein and tested the ability of this mutant *pfk1* gene to complement *Δpfk1* cells. I tried to define the relationship between PFK activity and its role in glycolysis with its possible role in peroxisome microautophagy via this experiment. The following sections are the results of these studies.

Degradation of Peroxisomes by Microautophagy Requires *PFK1*

PFK1 Gene Disruption and the Verification

A 5.6 kb *PFK1* "knockout" fragment was constructed by replacing an 1.2 kb Hind III fragment in *PFK1* with a 3.0 kb *S. cerevisiae ARG4* (Fig 5-1, upper and middle panel). PPF1 (*his4*, *arg4*) was transformed with this knockout fragment and stable transformants were selected on a transformation plate with histidine supplement but lacking arginine. We expected to find a 5.6 kb DNA fragment on Southern blots if *pfk1*::*ARG4* was inserted into the *PFK1* locus. In the parental strains and the *gsa* mutants, we found a 3.8 kb BamH I fragment that hybridized to our *PFK1* probe. However, the 3.8 kb BamH I fragment in the "knockout" WDKO1 (*his4*, *pfk1*::*ARG4*) had been replaced by the expected 5.6 kb BamH I "knockout" fragment (Fig. 5-1, lower panel). Interestingly, another transformant PPF11-1 had both 3.8 kb and 5.6 kb DNA locuses suggesting that the *pfk1*::*ARG4* did not incorporate into the *PFK1* site but instead into an unknown second site in this particular transformant. The 5.6 kb fragments were detected using a *S. cerevisiae ARG4* probe (data not shown). WDKO1 was thus named as *Δpfk1* or *Δgsa1*.

The *PFK1* Knockout Has the Same Phenotype as *gsa1-1*

The phosphofructokinase activity in WDKO1 was 15% of that measured in parental PPF1 (Fig. 5-1). This activity was comparable to that measured in *gsa1-1* mutant. The presence of normal *PFK1* in PPF11-1 was substantiated

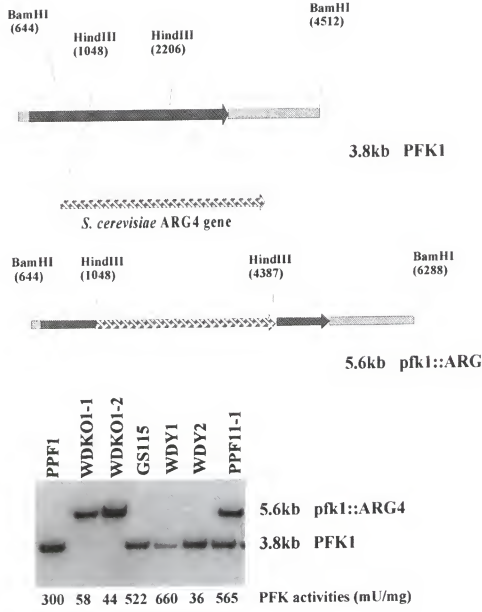


Figure 5-1. Generation of the *PFK1* knockout. The knockout fragment (5.6 kb) was prepared according to Materials and Methods. PPF1 was transformed with this fragment and stable Arg$^+$ transformants were isolated. The genomic DNA of the knockout (clones 1 & 2) and other cells were isolated and digested with BamH I for Southern blot. The expected 5.6 kb DNA band was present in WDKO1 and PPF11-1 cells. However, PPF11-1 cells also had the normal 3.8 kb DNA band. Cell extracts were prepared and phosphofructokinase activities measured in these strains. The values represent averages of three or more determinations and are expressed in units per mg of protein.

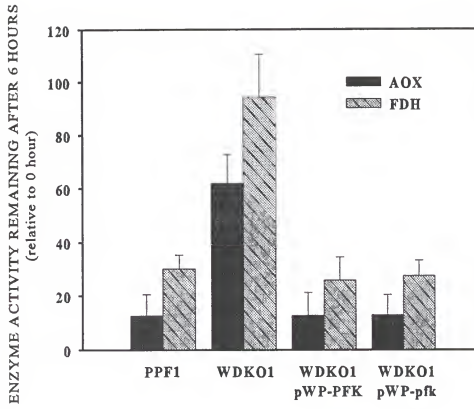


Figure 5-2. Glucose-induced degradation of AOX and FDH in PPF1 and WDKO1. PPF1 and WDKO1 were grown in methanol induction media then adapted to glucose. At 0 and 6 hours of adaptation, cell extracts were prepared and AOX and FDH activities measured. WDKO1 cells were transformed with pWP-PFK containing normal *PFK1* gene and pWP-pfk containing a mutated *pfk1* gene (see Materials and Methods). Stable transformants were isolated and their ability to degrade AOX and FDH during glucose adaptation determined. The values are presented as a percentage of the activities measured at 0 hour and represent the mean ± s.d. of three or more determinations.

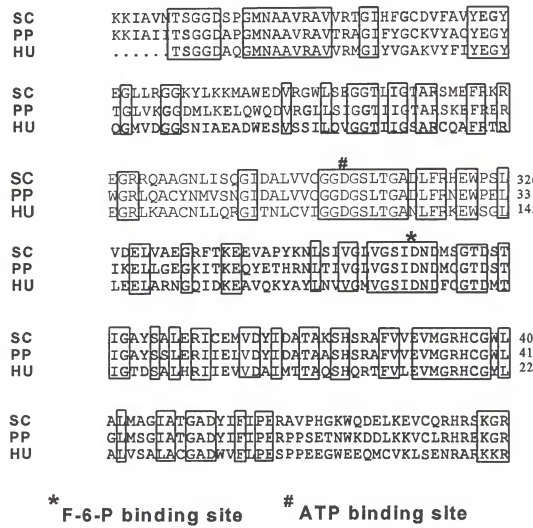
by its high levels of phosphofructokinase activity comparable to PPF1 (Fig. 5-1). We next compared the ability of PPF1 and WDKO1 to degrade AOX and FDH during glucose adaptation (Fig. 5-2). PPF1 and WDKO1 were grown in methanol and adapted to glucose medium for six hours. Over 80% of the AOX activity and 70% of the FDH activity were lost in PPF1. This rate of degradation was comparable to that observed in GS115. The degradation of AOX and FDH was significantly impaired in WDKO1. The amount of these enzymes remaining at six hours of adaptation was comparable to that seen for WDY2. The losses of both AOX and FDH in PPF11-1 that contained one wild type and one mutant copy of *PFK1*, were similar to PPF1 (data not shown). The result of this experiment showed that the WDKO1 mutants have the same phenotype as *gsa1-1*. Pfk1p is essential for the microautophagy of peroxisome.

Verification by Site Directed Mutagenesis of *P. pastoris PFK1* Gene of the Distinction between Rescue Function and PFK Activity

Normal *PFK1* and Catalytically-inactive *pfk1* Complement *Δpfk1*

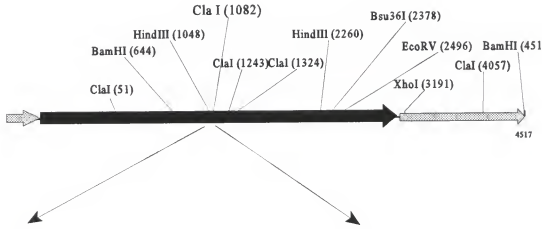
The *PFK1* knockout data suggested that Pfk1 protein is required for the onset of glucose-induced microautophagy. Since the identified primary role of PFK protein is to metabolize glucose, it is possible that Pfk1p is needed to produce a "signal" and initiates microautophagy. In order to evaluate whether

Partial Amino Acid Sequence Alignment of Phoshofructokinase



* F-6-P binding site # ATP binding site

Figure 5-3. Partial sequences comparison of Pfk1p in *P. pastoris* (PP), *S. cerevisiae* (SC) and *Human sapiens* (HU). The highest sequence homology of Pfk1 protein is seen in this region. The * site is the fructose-6-phosphate binding site of Pfk1 protein and is the main PFK enzyme active site. The # site is the major ATP binding site.



5'-GTT GGT TCT ATC GAT AAC GAT ATG TGC-3' DNA sequence

V G S I D N D M C Amino acid sequence

* *

-C GTT GGT TCT ATC AGT AAC GAT ATG TG- Primer sequences

- A CCA AGA TAG TCA TTG CTA TAC ACG C-

Asp362Ser Amino acid exchange

Figure 5-4. Site directed mutagenesis of *P. pastoris PFK1* gene. The upper panel of the figure is a restriction map of *PFK1* gene and its franking region. Important restriction enzyme sites are also included. The middle panel illustrates the site of mutation in the *PFK1* gene and the primer sequences constructed for site-directed mutagenesis. The Cla I (position 1082 from the beginning of the *PFK1*) was abolished due to the mutation and this was used as the first screen criteria for the right construction. Verification of mutation was done by sequencing from both directions. *Pfk1* was inserted into Xho I - Not I site in the pWP4 and this pWP-pfk1 was used to transform WDKO1 (*Δpfk1*) and *gsa1-1* cells. As a control, pWP-PFK1 was constructed and transformed into the mutant cells. *Indicates site of mutation.

the degradation of peroxisomes required catalytically active phosphofructokinase, I transformed WDKO1 with either *PFK1* or *pfk1* gene with its fructose 6-phosphate binding site inactivated (Berger and Evans, 1992, Arvanitidis and Heinisch, 1994). The full length of *PFK1* with 161 bases of its 5' flanking region was constructed into pYM4 (pWP-PFK). In a second construct, the aspartic acid at position 362 was changed to a serine by site directed mutagenesis as described in Materials and Methods (pWP-pfk) (Fig. 5-3 and 5-4). Both constructs were introduced into WDKO1 and *gsa1-1* cells. The integration was induced by linearization of the constructs at the *His4* gene site. Stable transformants were then subjected to AOX and FDH assays under glucose adaptation condition and PFK assay as described in Materials and Methods.

Phosphofructokinase activity was increased twofold in WDKO1 cells stably transformed with pWP-PFK. However, this activity was still significantly lower than that measured in parental PPF1 (Fig. 5-1). Since *PFK1* was directed to its *HIS4* gene locus and the 5' non-transcribing flanking region of *PFK1* includes only 161 nucleotides, it may not include the complete promoter and thus only allow low level transcription of *PFK1*. WDKO1 that had been stably transformed with pWP-pfk had only 50% of the phosphofructokinase activity measured in WDKO1. Thus, cells not expressing *PFK1* (*pfk1*::*ARG4*) have more phosphofructokinase activity than cells expressing a mutant pfk1 protein (*pfk1*::*ARG4*, *pfk1*).

Restoration of AOX Degradation Ability of *Δpfk1* by *PFK1*

We next examined the ability of *PFK1* and *pfk1* to restore the degradative capacity of WDKO1 *(Δpfk1)*. The glucose-induced degradation of AOX and FDH was dramatically slower in WDKO1 cells than in parental PPF1 cells. Substantial levels of both AOX and FDH remained in WDKO1 cells at 6 hours of adaptation (Fig. 5-2). When WDKO1 cells were transformed with either *PFK1* or *pfk1*, the degradation of AOX and FDH was comparable to PPF1 cells (Fig. 5-2). The data indicate that *PFK1* is required for glucose-induced microautophagy. However, this degradative event proceeds independent of its glycolytic activity. We propose Pfk1p is a bifunctional protein independently regulating glucose metabolism and glucose-mediate microautophagy.

Nonconventional functions have been attributed to many glycolytic enzymes (Smalheiser, 1996). In addition to their abilities to bind DNA and actin (Clarke, et al., 1985, Ronai, 1993), several enzymes have been shown to have nonenzymatic functions. For example, glucophosphoisomerase stimulates growth of neuronal tissues (Mizrachi, 1989). Interestingly, the trophic effects are found only when this enzyme is in the enzymatically inactive monomeric form. Neuron-specific enolase enhances neuronal survival and can be found within the nuclei of damaged nerves (Angelov, et al., 1994, Takei, et al., 1991). The redistribution of cytosolic enolase to the nucleus during neuronal repair was transient suggesting a role for this protein in

neuron growth. Robbins and coworkers reported that a single base mutation in glyceraldehyde 3-phosphate dehydrogenase alters endocytosis in CHO cells (Robbins, et al., 1995). In addition, this enzyme plays a role in neuronal apoptosis (Ishitani, et al., 1996). Cellular levels of glyceraldehyde 3-phosphate dehydrogenase have been shown to increase in dying neurons. However, if this increase is suppressed by the addition of antisense oligonucleotides, the neurons continue to survive. The mechanism of glyceraldehyde 3-phosphate dehydrogenase in these cellular processes is unknown, but appears to be unrelated to its glycolytic activity. Treptau et al. (1995) found phosphoglucomutase (PGM) underwent rapid dephosphorylation during trichocyst exocytosis in *Paramecium tetraurelia*. A protein phosphatase/kinase system was suspected to be involved in the modification of PGM and thus the regulation of exocytosis in *P. teraurelia* cells (Kissmehl, et al., 1997). The glycolytic enzymes have been long known to bind reversibly to the cytoskeleton (Arnold and Pette, 1968, Roberts and Somero, 1989). This binding leads to the activation of the enzymes and their close proximity to each other when bound to cytoskeleton provides a more efficient glycolytic flux (Clarke, et al., 1985). However, the effects of binding on actin structure and function have not been investigated. It will be interesting to investigate the possibilities that some of glycolytic enzymes or glycolysis products do have non-conventional functions that signal or regulate cell dynamic change under stress via processes such as endocytosis, exocytosis or autophagy.

Morphological Characterization of *Δpfk1*

We have shown that suppressed degradation of peroxisomes in *gsa1-1* cells was coincident with peroxisomes accumulating outside the yeast vacuole (Fig. 5-5). Thus, we propose that *GSA1* is required for an event upstream of vacuolar degradation, possibly the initiation of autophagy. If *GSA1* were *PFK1* as we expect, the morphology of WDKO1 (*Δpfk1*) during glucose adaptation should be indistinguishable from that observed for *gsa1-1*. This was indeed the case. In both *Δpfk1* and *gsa1-1* cells after 3 hour glucose adaptation, the peroxisomes appeared normal and were found outside the vacuole. The vacuole contained minimal cellular debris and was noticeably round with no invaginations or protrusions as seen in GS115. The indistinguishable morphological presentation of *gsa1-1* and *Δpfk1*, the similar biochemical presentations in these two strains and, the absence of PFK mRNA in *Δpfk1* (data not shown) suggested that *gsa1-1* is a null mutant of *PFK1*.

The *Δpfk1* cells were also transformed with pWP-PFK containing a full length *PFK1* and pWP-pfk with the PFK active site mutagenized. The morphologic observation was also carried out in these transformants. As shown in Fig. 5-5, In *Δpfk1* cells that had adapted to glucose for 3 hours, cells maintained large peroxisomes. The yeast vacuole was round and little cell debris presented. However, in *Δpfk1* and *gsa1* transformed with either pWP-PFK or pWP-pfk (Fig. 5-6), after three hour glucose adaptation, peroxisomes

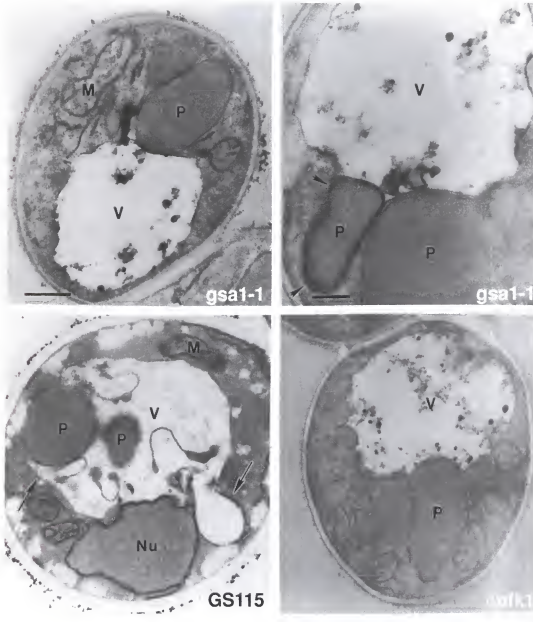


Figure 5-5. Morphology of GS115, *gsa1-1* and *Δpfk1* during glucose adaptation. GS115, *gsa1-1* and *Δpfk1* were grown on methanol and then adapted to glucose for 3 hours. Cells were prepared by a potassium permanganate fixation protocol for ultrastructural analysis (Tuttle et al.,1993). The vacuoles in *gsa1-1* and *Δpfk1* cells contained little cellular debris and finger-like protrusions seen in GS115 (arrows) were absent. Peroxisomes in *gsa1-1* and *Δpfk1* were found outside the vacuole. Occasionally, a peroxisome was seen to be surrounded by multiple membranes (arrowhead). Nu, nucleus; P, peroxisome; and M, mitochondria. Bars, 0.2 μM in upper right panel and 0.5 μM for all other panels.

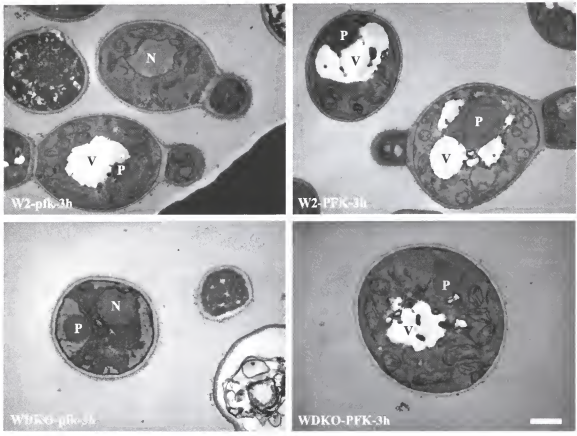


Figure 5-6. Morphology of WDKO1 (*Δpfk1*), WDY2 (*gsa1*) and its transformants during glucose adaptation. *Gsa1-1* cells and *Δpfk1* transformed with pWP-pfk and pWP-PFK were grown on methanol induction medium and then adapted to glucose for 3 hours. Cells were harvested, fixed in potassium permanganate, and prepared for electron microscopy study. In cells transformed with pWP-PFK or pWP-pfk, the yeast vacuole was active in wrapping the peroxisomes by its protrusions of vacuole membrane. In the left lower panel of *Δpfk1* cells transformed with site-directed mutagenized *pfk1,* a peroxisome is inside the vacuole and its membrane has been lost. In the upper right panel of *gsa1-1* cells transformed with normal *PFK1,* a peroxisome has been partially degraded. N, nucleus; P, peroxisome; and M, mitochondria; V, vacuole. Bar, 0.7 μM.

were wrapped by the protrusions of the yeast vacuole and some of peroxisomes were being degraded inside the vacuole in both transformants. No evidence of peroxisome macroautophagy was observed since no multiple membrane layers were seen around these peroxisomes which is the hallmark of macroautophagy. The biochemical data (Fig. 5-2) also support that the degradation of peroxisome proceeds normally in these mutants transformed with either *PFK1* or *pfk1* containing gene vectors. Our combined results suggested that PFK modulates microautophagy by a mechanism independent of its glycolytic activity.

Chapter Summary

To verify that Pfk1 protein is required for microautophagy, I generated a knockout strain *Δpfk1* with disrupted *PFK1* gene through homologous recombination of a 5.6 kb knockout fragment into the PPF1 genome. I examined the ability of *Δpfk1* to degrade AOX in response to glucose adaptation. As observed in *gsa1-1*, *Δpfk1* was unable to degrade AOX and FDH during glucose adaptation. In addition, the morphology of glucose-adapting *Δpfk1* cells was indistinguishable from that observed in *gsa1-1* cells. This verifies that Pfk1p is needed for the microautophagy. I also mutated the catalytic site of the Pfk1 protein and tested its ability to complement *Δpfk1* cells. I showed that despite its mutated catalytic site and the loss of PFK

activity in the transformant, *pfk1* complemented *Δpfk1* when analyzed by its AOX degradation ability. The morphological studies of *gsa1-1* and *Δpfk1* transformed with *PFK1* gene and *pfk1* gene also supported this view. In conclusion, I have shown that Pfk1p is required for the onset of glucose-mediated microautophagy of peroxisomes independent of its glycolytic activity. The questions remaining are 1) Does PFK2 has a role in the initiation of microautophagy? 2) What is the signal pathway of microautophagy and how does Pfk1p fit in there? 3) What other regulatory molecules are present in this process? I suggested that PFK modulates microautophagy by a mechanism independent of its glycolytic activity. It may either directly modulate autophagy via another yet to be identified functional domain or it may use its kinase domain to modified a substrate which in turn regulates the autophagy process. Further pursuit of *gsa1* as well as other *gsa* mutants will address these questions.

CHAPTER 6
CHARACTERIZATION OF *gsa7* MUTANTS

Introduction

A comparison study of glucose induced AOX and FDH degradation after methanol induction in *gsa7* and other mutants has shown in Fig. 3-4 in chapter 3. *Gsa7* showed impaired ability to degrade AOX and FDH in a six-hour glucose adaptation when compared with that of parental GS115 cells. The peroxisomal AOX and cytosolic FDH degradation in the ethanol adaptation pathway was not affected in *gsa7* (Table 3-1). In order to identify possible glycolysis mutants, I have tested some important glycolysis enzyme activities in selected *gsa* mutants and found *gsa7* was not involved in glycolysis steps (Fig. 4-11). *Gsa7* was chosen for subsequent morphologic and genetic studies. I will discuss the results in the following sections.

Morphological Studies of *gsa7*

Ultrastructure studies were also carried out in *gsa7* mutants. Several representative images of *gsa3* and *gsa7* mutants at zero- and three-hour

glucose adaptation are presented in Fig. 6-1. At zero hour of adaptation, large peroxisomes were induced due to the need for methanol metabolism enzymes in both *gsa3* and *gsa7*. The yeast vacuole was relatively round and small. No vacuolar membrane invagination and protrusion was observed. After three hours' glucose adaptation, *gsa3* and *gsa7* showed the projections of the vacuolar membrane, which sometimes wrapped the peroxisomes. However, no vacuolar membrane fusion was observed in *gsa3* and *gsa7* which bore similarity to that of the *gsa2* mutant (see morphological description in Chapter 4). We did not observe peroxisome degradation in the yeast vacuole in *gsa3* and *gsa7*. This was corroborated by our biochemical studies in these two strains that degradation of peroxisomal AOX was impaired. In fact, we believed that morphologically, *gsa2, gsa3* and *gsa7* belong to the same mutant group defective in the homotypic fusion event of microautophagy (see Chapter 3) and we believe that Gsa2p, Gsa3p and Gsa7p act downstream of Gsa1p in the glucose induced peroxisome autophagy pathway.

Recovery of *GSA7* Gene and its Verification

pYWP7-4 Rescues *gsa7* Phenotype

Gsa7 cells were transformed by electroporation with a genomic DNA library (see Materials and Methods and also Chapter 4). The putative rescue colonies of *gsa7* mutants that appeared white (AOX was degraded) on the filter

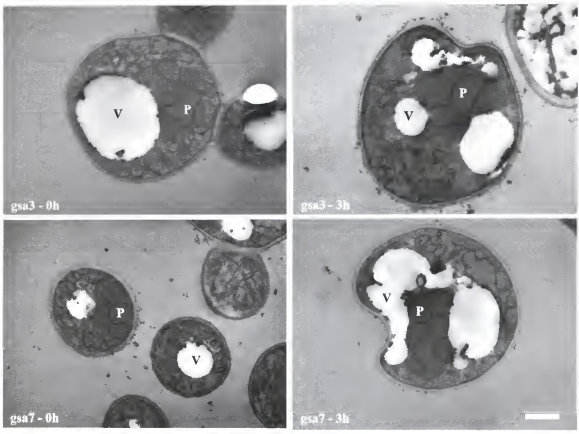


Figure 6-1 Morphology of *gsa3* and *gsa7* mutants during glucose adaptation. *Gsa3* and *gsa7* mutants were grown on methanol media for 24-36 hours and then were switched to glucose for adaptation. The cells were collected at 0 and 3 hours of adaptation and prepared by a potassium permanganate fixation protocol for ultrastructural analysis (see Materials and Methods). Cells at 0 hour showed large peroxisomes were induced. They were round and with little vacuolar activity. At 3 hours of glucose adaptation, the vacuolar protrusions of *gsa3* and *gsa7* wrapped around the peroxisomes but no vacuolar membrane occurred. Bar = 0.9 μm.

paper in a direct colony assay were collected. A total of 30,000 colonies were screened by this method. Thirty clones were collected from the direct colony assay screening for liquid media AOX assay verification. Only clone 1, clone 4 and clone 5 showed reproducible rescue ability by both assays. The three plasmids were recovered from these clones and were named pYWP7-1, pYWP7-4 and pYWP7-5 accordingly. The first two plasmids contain the overlapping genomic DNA fragment identified by restriction enzyme digestion. However, the insert in pYWP7-1 is 0.5 kb longer at the 5' end of the insert than that of pYWP7-4 which has about a 4 kb insert. pYWP7-5 has a 10 kb insert according to the estimation of restriction enzyme digestion. PCR of pYWP7-1, pYWP7-4 and pYWP7-5 with two primers generated from sequence inside pYWP7-4 insert yielded identical bands of predicted DNA fragment size (data not shown). Therefore, we concluded that all inserts in these three plasmids overlap. When these three plasmids were reintroduced into *gsa7* by transformation, all could rescue *gsa7* mutants. Only the rescue results of pYWP7-1 and pYWP7-4 were shown in Fig. 6-2.

Identification of *GSA7*

Once the rescue function of pYWP7-1 and pYWP7-4 and pYWP7-5 were verified, the 4.0 kb insert in pYWP7-4 was sequenced from both directions by DNA Sequencing Core of University of Florida. By searching the NCBI sequence database with the insert sequence, three open reading frames that code putative proteins were returned. A 104 amino acid of C terminal protein

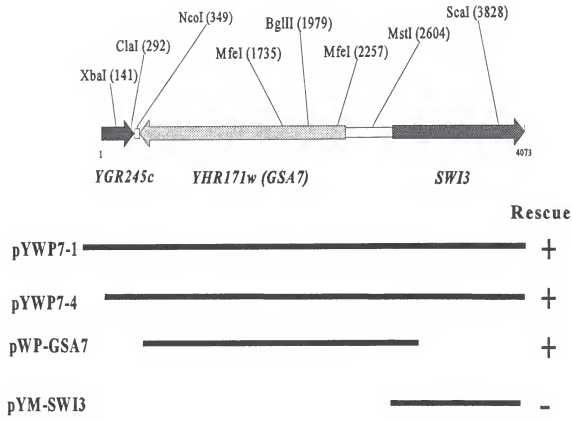


Figure 6-2 Verification of *GSA7*. The restriction map of pYWP7-1 insert is provided here. One full length ORF and two partial ORFs were found to be included in the insert. The pYWP7-4 and pYWP7-1 could rescue *gsa7* when reintroduced into *gsa7*. A DNA fragment containing complete putative *GSA7* gene showed rescue effect while the DNA fragment containing N-terminal half of the *Swi3* gene could not rescue *gsa7*. This further suggested that the full length ORF encodes our Gsa7p.

sequence that is homologous to *S. cerevisiae* Ygr245cp is at the beginning of the insert (see Fig. 6-2). A 340 amino acid of N terminal protein sequence that is homologous to Swi3 protein of *S. cerevisiae* is at the end of insert. In between is a full length putative protein that is homologous to *S. cerevisiae* Yhr171wp. Swi3p has been identified as a global transcription factor in many species while the functions of Ygr245cp and Yhr171wp have not been identified. In order to verify that the putative *P. pastoris* homolog of *YHR171w* gene is our *GSA7*, I subcloned *P. pastoris* DNA fragments containing *SWI3* and *YHR171w* genes into pYM8 (an episomal vector) or pYM4 (a non-episomal vector) respectively to study which gene was responsible for the rescue effect. The results of rescue study of these fragments were shown in Fig. 6-2. The DNA fragment in pYM4 containing *YHR171w* could complements *gsa7* but not the pYM8 containing *SWI3*.

Sequence Analysis of *GSA7*

The DNA and amino acid sequences of *GSA7* are shown in Fig. 6-3. *GSA7* gene encodes a putative 654 amino acid protein with a molecular weight of 71 kDa. A sequence comparison of Gsa7p with its homologs in yeast and human is shown in Fig. 6-4. As we can see, the Gsa7p and its yeast homologs in *S. cerevisiae* and *S. pombe* show greatest homology in the C terminal half of the proteins. It also showed high homology to a partial human sequence identified by searching EST database. Conceivably, this C-terminal region

```
1     AAAAAGAAAGATGCGGGGATGCGCAATAAGCTTTTCATTCACTGATGATAGGGTTGCGGATCTTCTTCCCAGAAGCCTGTTTTTTTTTTC

91    GCAAAAATCTTGAAAGACGTTTCCACTGGTAGGCTGTTGATATCTTCTAAACAGGCCAGATATCCCCCTTTTTAGTACGCTGAAAGCCA

181   AGCAGTTTACAGGAATGGCGTGGTCGTGTCAGAATCAAATGTCGCGTTAGGCCCCATCGAGCCTTCCCTATTTGTCTAACCTTTAGAAGG

271   TTCCTTCCCCCACACAGTAACCATGACAAGCATGGATATCCCCTATTCGCAAATAAGTTCATTTGTGAACTCGTCATTCTTCCAGAAGGT
                                  M  T  S  M  D  I  P  Y  S  Q  I  S  S  F  V  N  S  S  F  F  Q  K  V

361   GTCCCAATTGAAGCTCAACAAATATAGACTGGACGACACGGACAAGGCGATAGTGGGCAGTGTCGATTTTAAGTTCATTGGGAAGAACCA
121   S  Q  L  K  L  N  K  Y  R  L  D  D  T  D  K  A  I  V  G  S  V  D  F  K  F  I  G  K  N  Q

451   GCCAACAAGTTTATCTGTAGATGAGTCAAGCTTCAATGATAATATAACTTACACGCATGCACAGTTCCCGGTCAAAGGAATACTGAAGAA
151   P  T  S  L  S  V  D  E  S  S  F  N  D  N  I  T  Y  T  H  A  Q  F  P  V  K  G  I  L  K  N

541   CTTAAACACGGTTGAGGATTTCAGAAAGGTGGACAAGAATGAATTTCTTCAATCACAGGGTTTGGTGGTGCATAAATCAATTCAAGACCG
181   L  N  T  V  E  D  F  R  K  V  D  K  N  E  F  L  Q  S  Q  G  L  V  V  H  K  S  I  Q  D  R

631   TTCGTGTCTAAAAGATCTCTCAAAGTTGACCCAATTTTTCATTCTGTCCTTTAGCGACTTGAAAGGGTTCAAATTCATCTATTGGTTTGG
211   S  C  L  K  D  L  S  K  L  T  Q  F  F  I  L  S  F  S  D  L  K  G  F  K  F  I  Y  W  F  G

721   CTTCCCATCATTAGTGAGTAGATGGAAAGTAAACAAACTGAGTGGTCTAACTGAATCGCAGATAGAGCCGTATGAAAGTAAGCTTAACGA
241   F  P  S  L  V  S  R  W  K  V  N  K  L  S  G  L  T  E  S  Q  I  E  P  Y  E  S  K  L  N  E

811   GTGGCTAAATGCTCGTTTGCCCATTGAGCAAAAGCAAGCTTTCATTATTGACAACCTTGAATTCAAACCGTTTGAACAATTGTCAAGTTT
271   W  L  N  A  R  L  P  I  E  Q  K  Q  A  F  I  I  D  N  L  E  F  K  P  F  E  Q  L  S  S  F

901   TTCACCTGACGATCAGCTTAACATTGGGTTATTGACACCAGCAGCATCCTCAACAAATGTTCCACCCAGTTGAGAAATATTCTGTACAT
301   S  P  D  D  Q  L  N  I  G  F  I  D  T  S  S  I  L  N  K  C  S  T  Q  L  R  N  I  L  Y  M

991   GCTGGCTTATTATGGCTTTGAGAACATCAAAGTATACAATTTCAGATTCAACAATACCACATCCTTACATTAGACATCACTCTTGCTGA
331   L  A  Y  Y  G  F  E  N  I  K  V  Y  N  F  R  F  N  N  T  T  S  F  T  L  D  I  T  L  A  E

1081  GCCTCTTACTTCCGAGCCAAAAACAACAGGGTGGGAGAGAACTGCTCAAGGTAAGTTGGGCCCAAACTGGCCGATATAGGTCGTTTGGT
361   P  L  T  S  E  P  K  T  T  G  W  E  R  T  A  Q  G  K  L  G  P  K  L  A  D  I  G  A  L  V

1171  TGACCCTGCCCGCTTGGCTGACCAATCAGTTGATCTAAATTTAAAGCTGATGAAATGGAGAGTCATGCCTGAACTTGATCTGGATATCAT
391   D  P  A  R  L  A  D  Q  S  V  D  L  N  L  K  L  M  K  W  R  V  M  P  E  L  D  L  D  I  I

1261  AAAGAATAGTAAGGTTCTACTTCTCGGTGCTGGAACACTGGGAAGTTATGTCTCGAGAGTATTGCTAGGATATGGAGTTCGACACATTAC
421   K  N  S  K  V  L  L  L  G  A  G  T  L  G  S  Y  V  S  R  V  L  L  G  Y  G  V  R  H  I  T

1351  GTTTGTTGATAATGGTAAAGTTTCATTCTCTAACCCTGTTAGACAACCGTTGTTCAATTTTACAGATTGTTTGGAGGGAGGTGCTTCGAA
451   F  V  D  N  G  K  V  S  F  S  N  P  V  R  Q  P  L  F  N  F  T  D  C  L  E  G  G  A  P  K

1441  AGCCGAAACTGCAGCCAAAGCATTGAAATTAATTTTTCCGTTAATAACAAGCCAAGGATATAACTTGGAAGTACCTATGGCTGGACACCC
481   A  E  T  A  A  K  A  L  K  L  I  F  P  L  I  T  S  Q  G  Y  N  L  E  V  P  M  A  G  H  P

1531  GGTTACCGATGAAAAAAGACAGTATGAAGACTATCAAAGGTTAGTGACCCTAATAAAGGAACATGATGTAGTTTTTCCTTCTAATGGATTC
511   V  T  D  E  K  R  Q  Y  E  D  Y  Q  R  L  V  T  L  I  K  E  H  D  V  F  L  L  M  D  S

1621  AAGGGAAACGAGGTGGCTGCCTACCGTGCTTTGCAACGTTTTGATAAAATTTGCATCACTGCTGCATTGGGATTTGATTCATACCTGGT
541   R  E  T  R  W  L  P  T  V  L  C  N  V  F  D  K  I  C  I  T  A  A  L  G  F  D  S  Y  L  V

1711  AATGAGACATGGAAACTGTTTAATACCGAGCACATAGAAGCGGAAGAGAACTCTCACAGGCTGGGATGCTATTTCTGCAACGATATCAT
571   M  R  H  G  N  L  F  N  T  E  H  I  E  A  E  E  N  S  H  R  L  G  C  Y  F  C  N  D  I  I

1801  TGCTCCGAAAGATAGCACAACTGATCGAACTTTGGATCAAATGTGCACAGTGACCAGACCAGGGGTAGCCTTACTTGCTAGTTCTTTGGC
601   A  P  K  D  S  T  T  D  R  T  L  D  Q  M  C  T  V  T  R  P  G  V  A  L  L  A  S  S  L  A

1891  TGCGGAACTATTTGTTTCCATTCTTCAACACCCTCTAAAGAGTCATGCGCCTGCATCACTCCATGATAATGCCACAGTTCTTGGATGTTT
631   A  E  L  F  V  S  I  L  Q  H  P  L  K  S  H  A  P  A  S  L  H  D  N  A  T  V  L  G  C  L

1981  ACCACACAACTCCGAGGGTTCTTCACAATTTCGAAACCTCCAAACTTGAAGCAAATAACTACGAATACTGCTCTGCATGTTCGATACA
661   P  Q  Q  L  R  G  F  L  H  N  F  E  T  S  K  L  E  A  N  N  Y  E  Y  C  S  A  C  S  I  Q

2071  GGTATTAAACGAATACAAATCCAGAACTTGGGATTTGTCAAAGATGCCCTGAATGAAAACAATTATCTTGAGGATTTGACAGGCCTTAC
691   V  L  N  E  Y  K  S  R  T  W  D  F  V  K  D  A  L  N  E  N  N  Y  L  E  D  L  T  G  L  T

2161  TAAGGTCAAGCAAGAATCTGAGATAGCCGAGAAGAAGTTTCAAGAGTTTGAAAACGGTTTAGAGTTTAGTGATGAAGATTCAGAATGGAT
721   K  V  K  Q  E  S  E  I  A  E  K  K  F  Q  E  F  E  N  G  L  E  F  S  D  E  D  S  E  W  I

2251  AAACTAATATACAAAAACTAATCCATGGGGTCTGGAAAATTAAGAAAAAATATATTTAATGACCCTTCTTTTTTGCTTATCGATATGA
751   N  *

2341  GCTCGTAGAACCTTTTGCTTATCACGAAGAGCCATTTTCTGTTTGCTTTGCACACCTCCTTGTGGATGGTCATCAAGAAGTTCTTCTTA

2431  CGAGCCTTCTCTTTATTAGTGGTAGAATGCTCTCCTTCTCTCTTTCCTCTCTAGAACCATGAGCATCTTCATCACGACCAGCCTTGACA
```

Figure 6-3 Complete nucleotide and amino acid sequence of *GSA7*. *GSA7* gene encoded a 654 amino acid, 71.2 kD putative novel protein. No known protein motifs were recognized by our motif search.

```
Gsa7      MTSMDIPYSQIS..SFVNSSFF.........QKVSQLKLNKYRLDDTDKAIVGSVDFKFI   49
Yhr171w   -s-ervlsyapafk--ldt---.........-el-r---dvlk--s-cqpltvnl-ihn-   51
S.pombe   -fvgkalqf-sfhs-idatfwhqlsnykvek--ldasp-tihgkfn-ysrgni-iv-gea   60

Gsa7      GKNQPTSLSVDES.SFNDNITYTHAQFPVKGILKNLNTVEDFRKVDKNEFLQSQGLVVHK  108
Yhr171w   p-sadqvplfltnr--ekhnnkrtnev-lq-sif-f-vlde-knl--ql--hqra-ecwe  111
S.pombe   ps-snikdcla-gtll-a-tpqeftnad--k-reeige-llnsiknqvvserpne-lrfl  120

Gsa7      SIQDRSCLKDLSKLTQFFILSFSDLKGFKFIYWFGFPSLVSRWKVNKLSGLTESQIEPYE  168
Yhr171w   dgi.....--in-cvs-v-i--a---kyr-y--l-v-cfqrpsstvlhvrp.....--sl  161
S.pombe   ifsyadikaykyhywcl-p.--ketphwivkdlspae--ip.......--pil---refl  172

Gsa7      SKLNEWLNARLPIEQKQAFIDNLEFKPFEQLSSFSPDDQLNIGFIDTSSILNKCSTQLR  228
Yhr171w   kg-fskcqkwfdvnyskwvc-ldaddeivnydkciirktkvlairdtstmenvpsaltkn  221
S.pombe   -tadyyqrpfflliksktldewtiapl-elshcvdk-lqfy-vaedsvqlaeypswpv..-  230

Gsa7      NILYMLAYYGFENIKVYNFRFNNTTSFTLDITLAEPLTSEPKTT.............GW  274
Yhr171w   flsvlqydvpdlidfkllliirq-eg--a-na-f-sidpqsssssn........pdmkvs--  273
S.pombe   ---afafikfklkvinlflyrdgin-d--sksilikveadkdmileaplsivgwerngkg  290

Gsa7      ERTAQGKLGPKLADIGALVDPARLADQSVDLNLKLMKWRVMPELDLDIIKNSKVLLLGAG  334
Yhr171w   --nv----a-rvv-lss-l--lki---------------il-d-n------t--------  333
S.pombe   v.....---rvvnlstvl--fv-sesast---s--r--lv-q----r-q---c------  344

Gsa7      TLGSYVSRVLLGYGVRHITFVDNGKVSFSNPVRQPLFNFTDCLEGGAPKAETAAKALKLI  394
Yhr171w   ---c----a-iaw---k-------t--y------a-y--e--gk.....-l--as--r-  390
S.pombe   ---cg-a-n--sw----v----yst--y------s--t-e--krkl.---c--qr--e-  403

Gsa7      FPLITSQ.GYNLEVPMAGHPVTDEKRQYE..DYQRLVTLIKEHDVVFLLMDSRETRWLPT  451
Yhr171w   ---mdat.-vk-si--i--klvn-ea-hk..-fd--ra------ii---v----s----s  447
S.pombe   y-nmkfst---isi--i---iyeagiektmh--et-en--st--ai---t-t--s-----  463

Gsa7      VLCNVFDKICITAALGFDSYLVMRHGNLFNTEHIEAEENSHR.....................  493
Yhr171w   l-s-ien-tv-n-----------------rdeqsskq.........................  482
HGsa7     ...........in-----tfv-----lkkpkqggagdlcpnhpvasadllgsslfanipg    49
S.pombe   -istam--ll-ns------w------svlqk-n........-igcyfcndif.........  507

Gsa7      ..LGCYFCNDIIAPKDSTTDRTLDQMCTVTRPGVALLASSLAAELFVSILQHPLKSHAPA  551
Yhr171w   ..------h-vv--t--l-----------------mm-----v--mt-l--tkysgset.  539
HGsa7     yk--------vv--g---r------q---s---l-vi-ga--v--m--v----eggy-lp  109
S.pombe   ............--sn-lv------t-----s-c-ni-tai-v-----l----ng.--ap  554

Gsa7      SLHDNATV....LGCLPQQLRGFLHNFETSKLEANNYEYCSACSIQVLNEYKSRTWDFVK  607
Yhr171w   .....-----.--di-h-i------sil---tpa--h-p---pk-ieaftdlg-e---  589
HGsa7     aavtigmslqpl---ah-i.----sr-dnvlpvslafdk-t---sk--dq-eregfn-la  168
S.pombe   v-nedq--lge....--h-j-------slm-isgma-pq-----ecii--            600

Gsa7      DALNENNY.LEDLTGLTKVKQESEIAEKKFQEFENGLEFSDEDSEWIN              654
Yhr171w   k--ehpl-.--e                                                   600
HGsa7     kvf-sshsf------llh.--tqa--iwdmsddeti                          204
```

Figure 6-4 A protein sequence comparison of *GSA7* in *P. pastoris*, *S. cerevisiae*, *S. pombe* and *H. sapiens*. The C terminals of the four proteins showed highest homology to each other. The total protein sequence identity is 44% and similarity is 66% in yeasts. The motif search showed no significant homology to known protein motifs. However, the C terminal half of the protein has homology to *S. cerevisiae* Ubiquitin-activating enzyme. (.....) represent gaps between the gene. (-----) represent identity.

should be the main functional domain of the protein and thus the focus of our future study. Interestingly, this is also the region that has homology to ubiquitin-activation (UBA1) enzyme of *S. cerevisiae* . A protein motif search of Gsa7p and their other yeast counterparts using Prosite program failed to yield any active site or signature sequence that has been identified in UBA1 in yeast or human.

Δgsa7 Generation and its Phenotype Studies

I have shown that *P. pastoris YHR171w* complement *gsa7*. I next constructed a gene knockout fragment of *GSA7* gene and used it to knockout the *GSA7* in a double auxotroph parental strain PPF1. A Hind III - Bgl II fragment in *GSA7* were replaced by a *S. cerevisiae Arg4* gene (see Fig. 6-5, upper panel). A double auxotroph PPF1 (his4, arg4) were transformed with the knockout fragment (5.1 kb, cut out by Apa I and Sca I) by electroporation. The histidine auxotrophs grown on the transformation plates were chosen and direct colony assay of AOX were applied as a screening method. The colonies appearing purple is defective in degradation of AOX and thus the possible knockout mutant. Three clones in this assay showed purple color and were selected. The genomic DNAs of two clones along with GS115, *gsa7* and two other non-rescue transformants C3 and C4, were extracted for PCR screening. The primers used in PCR are depicted in Fig. 6-5. The 5' primer is inside the knockout fragment and precedes 350 bases of the start code of *GSA7* while 3' primer is outside the knockout fragment and just behind the stop codon of *GSA7* gene. In a true knockout strain, the PCR fragment should be 4.9 kb while in control strains, the

fragment should be 2.5 kb. The lower panel of Fig. 6-5 showed the PCR results of these strains. *Δgsa7-1* and *Δgsa7-2* have the predicted 4.9 kb fragment size but without the 2.5 kb fragment and thus the true knockout. The *Δgsa7s* were then subjected to AOX liquid media assay during glucose adaptation. As expected, the *Δgsa7*s lacked the ability to degrade AOX thus mimic the phenotype of *gsa7* while all other strains tested show normal ability to degrade AOX. This experiment showed that the gene we cloned is necessary for the microautophagy of peroxisomes and thus is Gsa7p.

A HA Tagged Gsa7p Rescues *P. pastoris*

To study the Gsa7 protein function of *P. pastoris*, I also needed to verify that Gsa7p is expressed in *P. pastoris*. I also wanted to identify the localization of the protein in the cells. I designed two primers flanking the *GSA7* gene for PCR purpose. The 5' primer is 350 bases upstream of the start codon to include its own promoter while the 3' primer is at the end of *GSA7* gene containing a HA epitope tag sequence. The *GSA7-HA* gene was amplified by PCR constructed into a pYM4 vector (see Materials and Methods). The HA tagged *YHR171w* gene (a homolog of *GSA7* in *S. cerevisiae)* was also amplified by PCR *of S. cerevisiae* genomic DNA (provided by Ms P. Wu) using the same strategy as in *P. pastoris*. The PCR products were then constructed into pYM4. Both vectors were introduced into *gsa7* and stable transformants were isolated. The

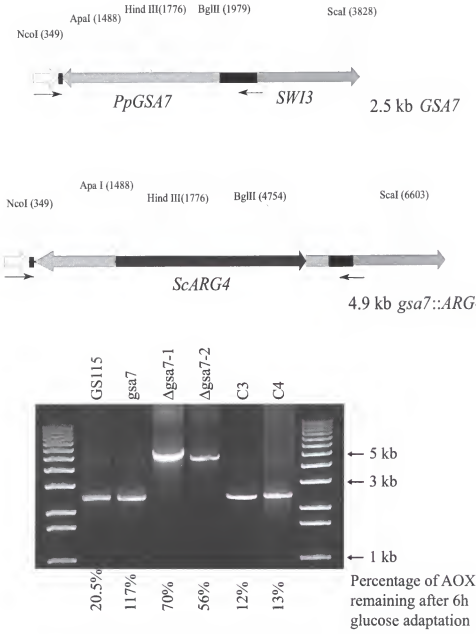


Figure 6-5 *Δgsa7* generation and its verification. A 5.1 kb *GSA7* knockout fragment was prepared (see text). PPF1 was transformed with this fragment and Arg$^+$ transformants were isolated and screened with an AOX direct colony assay. The genomic DNA of the knockouts (*Δgsa7-1* and *Δgsa7-2*) and other cells were isolated and their PCR results with two primers (see text) were shown in the lower panel. The expected 4.9 kb PCR products were present in *Δgsa7-1* and *Δgsa7-2* while absent in all other strains. The cell extracts of these strains were prepared and their AOX activities were measured. The values represent averages of three or more determinations and are expressed as a percentage of zero hour activity.

expression of both gene products in *P. pastoris* was confirmed by Western blot with an anti-HA epitope tag antibodies (Fig. 6-6). Clones P6B, P3B and P3G were *gsa7* transformed with a HA tagged *GSA7* gene of *P. pastoris* while clones S1, S3, S4 were *gsa7* transformed with a HA tagged *YHR171w* gene of *S. cerevisiae*. As shown in Fig. 6-6, no HA tagged protein was detected in clone P6B, S1 and *gsa7*. The HA tagged Gsa7ps were detected by the HA antibodies in all other clones at the predicted size of 71.1 kDa. When these clones were tested for their ability to degrade AOX activity by glucose adaptation assay, I found clone P6B and S1 were defective in degradation of AOX during a six hour glucose adaptation while all other clones regained their ability to degrade AOX during glucose adaptation. This is well correlated with the data that Gsa7p or Yhr171wp were expressed in these rescue clones. Interestingly, our preliminary result showed that, when clone P3B, P3g and S3 were grown on methanol and then adapted to glucose, the protein levels in these three clones did not change significantly at 0, 1 and 6 hour time point (data not shown). Our preliminary data suggested that Gsa7p is a cytosolic one. However, the location of the Gsa7p still need to be confirmed by immunofluorescence study.

Chapter Summary

In this chapter, I described the characterization of *gsa7* mutant as well as the identification of the *GSA7* gene. *Gsa7* is defective in peroxisome

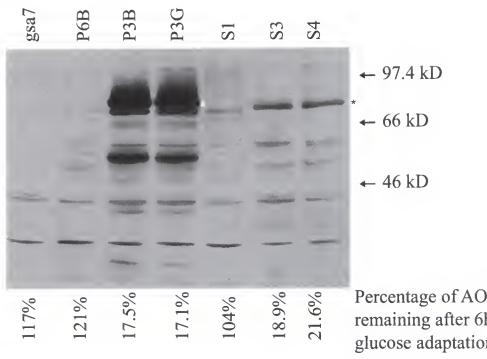


Figure 6-6 Detection of a HA epitope tagged Gsa7p and Yhr171wp in transformed *gsa7*. HA epitope tagged *GSA7* and *YHR171w* were constructed into pYM4 with their endogenous promoter and introduced into *gsa7*. The transformed *gsa7* cells were grown on YPD overnight and cells were prepared for Western blot analysis (the upper panel). Both Gsa7p and Yhr171wp have a predicted molecular mass of 71 kD. However, Gsa7p is higher than Yhr171wp on the Western blot with a molecular mass difference of 2-4 kD to that of Yhr171wp. These strains were also tested their ability to degrade alcohol oxidase (AOX) after a six hour glucose adaptation. The values presented are the percentage of AOX activity remaining after a 6 hour glucose adaptation. They were measured at least three times and presented as a mean of the total measurements. #Gsa7p; *Yhr171wp.

microautophagy. However, the ethanol induced macroautophagy pathway is intact. Our ultrastructural analysis showed that *gsa7* is defective in the an event of homotypic fusion of yeast vacuolar membrane in the autophagy process similar to *gsa2* and *gsa3* (see chapter 3). The functional complementation study of *gsa7* mutants with a genomic DNA library yielded a plasmid with an insert of 4.0 kb that rescued the mutant phenotype. Sequencing of this insert revealed a possible complete ORF of *GSA7* coded a 71.1 KD protein that is homologous to Yhr171wp of *S. cerevisiae*. Rescue study with different fragments of the insert verified that this putative *GSA7* in the insert could rescue *gsa7*. The *GSA7* gene knockout experiment showed that *GSA7* is essential for the peroxisome microautophagy during glucose adaptation. The HA epitope tag sequence was attached to the end of *GSA7* and *YHR171w* gene and introduced into *gsa7* mutants. The Western blot confirmed the expression of HA tagged Gsa7p and Yhr171wp in the transformed *gsa7* cells and these cells regained their ability to degrade AOX. This verified that the function of Gsa7p is conserved in *P. pastoris* and *S. cerevisiae*. Since microautophagy has not been observed in *S. cerevisiae* while macroautophagy has been identified in *S. cerevisiae* and our *GSA7* gene appeared to control a step of homotypic vacuolar membrane fusion in microautophagy, it will be interesting to knockout *YHR171w* gene in *S. cerevisiae* and to observe its phenotype and compare with that of *gsa7*. Further study of this protein in these two yeast strains will yield more information about the functional role of Gsa7p.

The discovery of Gsa7p and its verification give us an opportunity to examine for the first time a specific molecule that controls one of the important steps in the peroxisome autophagy process. We can do a whole array of experiments that previously not possible. *GSA7* gene and its *S. cerevisiae* counterpart *YHR171w* gene with a HA epitope tag have been put into pHWO10 vector behind a GAP promoter and the pPIC3 vector with an AOX gene promoter (Both vectors were provided by Dr. J. M. Cregg). Important information such as the relationship of vacuolar fusion genes can be derived if overexpression of Gsa7p in these plasmid could rescue *gsa2* and *gsa3, gsa6* and the other fusion mutant *gsa5.* We can also construct *GSA7* gene into a GAPDH promoter driven plasmid or an AOX prompter driven plasmid that we have and observe the overexpression of this protein in both wild type GS115 and mutant *gsa7.* We can also test if the expression or overexpression of the protein could rescue *gsa7* or other *gsa* mutants in the same fusion defective group such as *gsa2* and *gsa3.* Such experiments could probe the action sequence of these genes in the homotypic fusion event. Since no significant protein motifs were recognized by our gene database search, we could also use random PCR mutagenesis to mutate the *GSA7* gene. The products can then be introduced into *gsa7* and use AOX direct colony assay to identify those clones that could not rescue *gsa7.* Such clones should contain plasmid with mutated *GSA7* at critical protein functional sites. Sequence analysis of the plasmid in such clones will give us information as to which domain might be important to the protein function. The

C-terminal half sequence of the human *GSA7* homolog has been sequenced and we obtained the clones possibly containing the whole human *GSA7* gene (purchased from ATCC). We knew that this C-terminal half bears the highest homology throughout species and has sequence homology to ubiquitin-activating enzyme in *S. cerevisiae*'s. Sequence analysis of this human *GSA7* gene, further study of this gene expressing in *P. pastoris* mutants or *vice versa* and the functional study of this gene product in mammalian cell system will yield more information as to how this Gsa7p works in the autophagy process in both yeast and possibly mammalian cells system. Drs Oshumi and Klionsky's groups recently found that *S. cerevisiae*'s Gsa7p might act with other novel ubiquitin-like pathway enzymes and responsible for the defect in their autophagy mutants (personal communication). It is important to test the possible role of our Gsa7p's in this alternative pathway and if possible, to identify other players in this process.

CHAPTER 7
CONCLUSIONS AND PROSPECTS

Introduction

Protein degradation in eukaryotic cells occurs in all subcellular compartments. It is complicated in that there are many pathways for degradation of intracellular proteins (Olson et al., 1992). One reason for this diversity is the variety of intracellular compartments in which proteins are degraded. In addition, various proteins need to be degraded at different rates depending on the physiological state of the cell. The lysosomal protein degradation pathway is one of such pathways in mammalian cells. The functional equivalent of lysosome in yeast is its vacuole. Proteins or cell organelles are delivered to the yeast vacuole for degradation in a similar manner as in mammalian cells (Takeshige, et al, 1992, Dunn, 1994, Chiang, et al., 1996).

Our lab has characterized a system in methylotrophic yeast *P. pastoris* to investigate protein degradation by observing the process of peroxisome degradation in the yeast vacuole (Tuttle, et al., 1993). The general pathways of peroxisome induction and degradation under different carbon sources in *P. pastoris* are depicted in Fig. 7-1. When *P. pastoris* cells are grown on glucose,

they maintains a few small peroxisomes. However, when cells are grown on methanol, large peroxisomes are induced. During the subsequent growth carbon source switch such as glucose or ethanol, peroxisomes underwent microautophagy and macroautophagy respectively (Tuttle and Dunn, 1995). A simplified illustration of the concept of microautophagy and macroautophagy is provided in Fig. 7-2. In yeast, microautophagy is a process that peroxisomes or other cell components are engulfed by vacuolar membrane protrusions and the contents get degraded thereafter. Macroautophagy refers to peroxisomes or cell components are surrounded first by several layers of membrane to form autophagosomes and the autophagosomes then fuse with the yeast vacuole to release the contents of autophagic bodies for degradation. Based on previous work in this lab, we proposed the microautophagy process includes glucose signaling, peroxisome sequestration, vacuolar membrane homotypic fusion and vacuolar degradation of peroxisomes (see Fig. 7-4) and specific regulatory molecules needed to be synthesized to modulate or coordinate this autophagy process (Tuttle and Dunn, 1995). In order to investigate the mechanism of microautophagy, we have generated glucose-induced selective autophagy (*gsa*) mutants that are unable to degrade peroxisomal AOX during glucose adaptation. By investigating the biochemical and morphological phenotypes as well as genetic characteristics of *gsa* mutants, we tried to understand the regulatory mechanism of glucose induced microautophagy in this yeast. My studies consists of three parts. The first part is the characterization of *gsa* mutants.

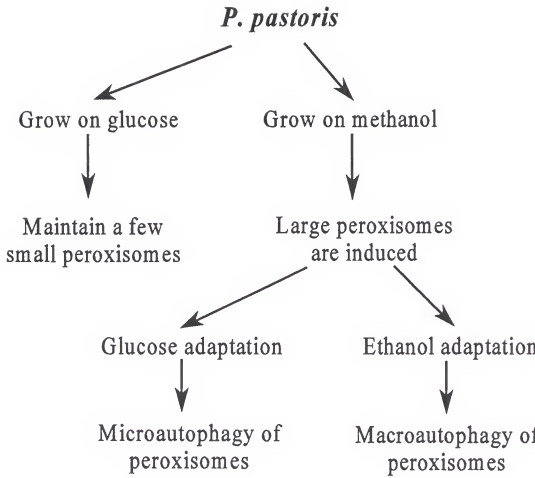


Figure 7-1 Pathway of peroxisome induction and degradation under glucose and ethanol adaptation in methylotrophic yeast *Pichia pastoris*. Under glucose growth *condition, P. pastoris* maintains minimum peroxisomes. However, under methanol growth condition, large peroxisomes are induced. During the subsequent adaption with either glucose or ethanol, cells undergo microautophagy and macroautophagy respectively to degrade peroxisomes.

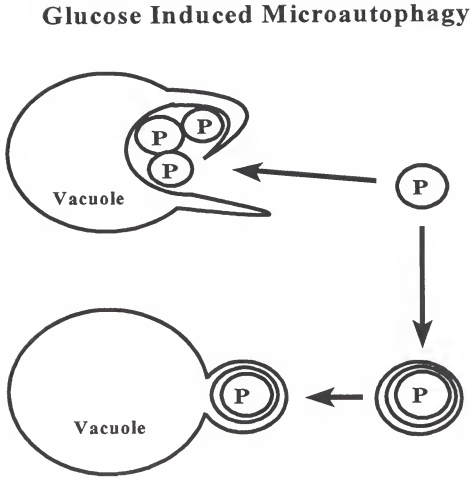


Figure 7-2 Microautophagy and macroautophagy of peroxisomes in *P. pastoris*. The simplified figure is used to illustrate the concept of microautophagy and macroautophagy of peroxisomes during nutritional adaptation. *P. pastoris* is grown on methanol till stationary and glucose or ethanol is added to begin adaptation. Upon glucose addition, peroxisomes undergo microautophagy while during ethanol adaptation, peroxisomes undergo macroautophagy in *P. pastoris*.

The second is the genetic study of *gsa1-1* mutant. The third is the genetic study of *gsa7* mutants. The studies I have finished provide insights on the poorly understood phenomenon of yeast microautophagy process.

Characterization of *gsa* Mutants

The characterization of *gsa* mutants included biochemical analysis of alcohol oxidase and formate dehydrogenase and the morphological characterization. Well-characterized mutants were then subjected to backcrossing and complementation analysis and were used for further genetic studies. I have characterized most of the available mutants in our stocks, and eight *gsa* complementation groups (*gsa1* to *gsa8*) have been identified. All *gsa* mutants lacked the ability to degrade peroxisomes during glucose adaptation. However, their ability to degrade peroxisomes during ethanol adaptation was not affected. Morphological studies of *gsa* mutants revealed that they have distinct phenotypes. *Gsa1 (pfk1)* is defective in the initiation of microautophagy (see Fig. 7-4). *Gsa4* and *gsa8* are defective in AOX and peroxisome degradation. However, their cytosolic protein degradation pathway seems intact. I believe they are defective at a step of peroxisome recognition in the microautophagy. *Gsa2, gsa3*, *gsa6* and *gsa7* are defective in a homotypic fusion step of the vacuolar membrane. In *gsa5*, macroautophagy was turned on during methanol induction and glucose adaptation, however, the degradation of peroxisome

under glucose adaptation via microautophagy was defective. I believe it also belongs to the group of homotypic fusion mutants but *gsa5* may inversely control a different step in the event of vacuolar membrane fusion than *gsa2, gsa3, gsa6* and *gsa7*. Recently Subramani's lab (Sakai et al., 1998) also generated peroxisome degradation mutants using the same method we developed (Tuttle and Dunn, 1995). However, they used a parental strain containing a constitutively expressed GFP-SKL protein to help in visualizing yeast peroxisomes. They have identified 6 peroxisome microautophagy (*pag*) mutant complementation groups. It will be interesting to see if any of *pag* mutants overlaps with our *gsa* mutants.

The biochemical and morphological phenotypes revealed in these studies indicate that the defective genes in these *gsa* mutants regulate different microautophagy steps that I have defined in the introduction of this chapter (also see Fig. 7-4). These simple but powerful tools can be used to further characterize the remaining *gsa* mutants as well as the functional roles of the corresponding gene products and thus the mechanism of microautophagy.

Regulation of Signaling of Microautophagy by Gsa1p

The *gsa1-1* mutant is the first mutant we chose for further genetic study. A complete *PFK1* sequence was recovered from plasmid that rescued *gsa1-1*. *PFK1* mRNA was greatly reduced in *gsa1-1* and so was its PFKp activity. This

showed that *gsa1-1* is *pfk1* mutant. Our *PFK1* knockout experiment confirmed that *PFK1* is essential for the onset of autophagy. Our site-directed mutagenesis of *PFK1* active site experiment suggested that PFKp activity is not needed for the initiation of autophagy.

PFKp is a highly regulated protein with many conserved domains capable of binding ATP, GTP, AMP, citrate, fructose 1,6-bisphosphate as well as actin. This protein is the major regulatory checkpoint allowing the cell to respond to changes in glucose concentrations. Our data suggested that the regulation of glycolysis and microautophagy by glucose is modulated by the α-subunit of phosphofructokinase. At this time, we have yet to define the role for this protein in microautophagy, and we have not observed any role for the β-subunit of phosphofructokinase in this process. Therefore, we do not know if microautophagy is mediated by the heterooctomeric phosphofructokinase complex composed of both α and β subunits or by monomeric α-subunit. We can only speculate that phosphofructokinase or its α-subunit may directly interact with either a vacuolar or a peroxisomal membrane protein thereby initiating the sequestration event. As an alternative hypothesis, Pfk1 protein might either add a phosphate to a substrate other than fructose-6-phosphate, which in turn regulates the microautophagy process or it may modify a sugar on a glycoprotein and this modified protein subsequently regulate the homotypic fusion of vacuolar membrane in the autophagy process. We can mutagenize the ATP/ADP binding site (Fig 7-3) that is adjacent to fructose-6-phosphate

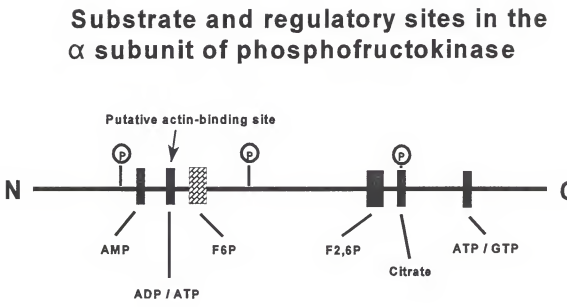


Figure 7-3 Putative substrate binding and regulatory sites in Pfk1p. This map showed important substrate and regulatory sites in the alpha subunit of phosphofructokinase. These sites are conserved across species. The putative Ser/Thr phosphorylation sites are also included.

binding site to see if the association of ATP to Pfk1p is important for autophagy. If the elimination of ATP binding site in PFK1 prevented the autophagy, the kinase activity of PFK protein might be important for the autophagy. We can also generate a *PFK2* knockout and observe if the microautophagy is normal in *Δpfk2*. if microautophagy were defective in *Δpfk2* mutant, then PFK protein is important in this process. We should compare PFK mRNA levels in all three *gsa1* strains and if possible, identify the mutation sites in *gsa1* mutants. Another way is to knockout the GAPDH gene downstream of PFKp action site in glycolysis (see Fig 4-9). If blockage of this glycolysis step in *P. pastoris* has no effect on autophagy, then Pfk1p is more likely to directly regulate the autophagy process. We can also generate random mutations in *PFK1* by PCR and then construct the PCR products into pYM8 and, transform *gsa1* with the plasmid. We can use direct colony assay to identify plasmid- containing colonies that do not rescue *gsa1*. Sequencing these plasmids may reveal sites or motifs in Pfk1p that regulate the autophagy process.

Regulation of a Homotypic Vacuolar Fusion Event of Microautophagy by Gsa7p

Gsa7 is defective in peroxisome degradation. It is a mutant which we believe is defective in homotypic fusion of vacuolar membrane. The morphological studies of *gsa7* supported that a step in the fusion event of vacuolar membrane is defective during the engulfment of peroxisomes. When

gsa7 cells were transformed with a *Pichia pastoris* genomic library, three overlapping inserts in pYM8 were recovered. Their complementing effect was verified by reintroducing them into *gsa7* cells. The sequence reveals one complete open reading frame and two partial ORFs. I have confirmed that only the DNA fragment with complete ORF rescue *gsa7*. The putative Gsa7p (encoded by the complete ORF) is a 654 amino acid protein with no identified function. The protein has homologs in *S. cerevisiae*, *S. pombe*, *C. elegans* and *H. sapiens* and its C terminal half showed homology to ubiquitin-activating enzyme. The *GSA7* gene knockout studies verified that *GSA7* is essential for the microautophagy process. By transforming *gsa7* cells with a HA epitope tagged full length *GSA7* gene or its yeast counterpart, *YHR171w* with their endogenous promoter, I showed that Gsa7p and Yhr171wp were expressed in *gsa7* and could rescue *gsa7*.

We knew Gsa7p is important for the homotypic fusion event in the autophagy. How Gsa7p does that? One speculation is that it participates in a novel ubiquitin-like protein degradation pathway (see chapter 6) and the degradation of a particular protein is important for the homotypic fusion of vacuole membrane. It is also possible that the ubiquitination of a regulatory protein leads to targeted vacuolar degradation of peroxisomes. Further studies of Gsa7p such as to mutagenize the putative ubiquitin binding cysteine, will yield information as to how Gsa7p acts in the cells, who else are its partners and where the protein functional domain of Gsa7p locates.

Prospects and Conclusion

In recent years, great progress has been made in understanding the mechanisms by which proteins and organelles are delivered to the vacuole from the cytoplasm. Various independent studies and approaches have been conducted and applied in the studies of protein and cell organelles transport from the cytoplasm to the vacuole (Klionsky, 1997). However, many questions remained unanswered. How are the signals for autophagy transduced? How is membrane binding or engulfment of a substrate initiated and carried out? Are v- and t-SNAREs and rab proteins involved in the targeting and delivering process? How do cells sense the stop signals of autophagy and transduce them to the related cell organelles? Although my study did not resolve all the questions, significant progress has been made due to the isolation of two *GSA* genes that play a role in the microautophagy process.

As stated in the introduction chapter, peroxisomes are induced by growing cells on methanol. In the subsequent glucose adaptation, cells undergo rapid degradation of peroxisomal and cytosolic proteins. This process is similar to microautophagy that has been defined in mammalian cell system. It also needs molecules synthesis to regulate and coordinate this process (Tuttle and Dunn, 1995). The microautophagy process of peroxisomes generally consists of glucose signaling, vacuole recognition and peroxisome sequestration, homotypic vacuolar membrane fusion and finally, the vacuolar degradation of peroxisomes

(Fig. 7-4). *Gsa* mutants have been generated to identify the regulatory molecules in the peroxisome microautophagy. The glucose induced peroxisome microautophagy and it relationship with *gsa* mutants is shown in Fig. 7-4. Currently, I have identified eight *gsa* complementation groups with distinct characteristics. A gene that is important for the autophagy process is defective in each of my *gsa* mutants. Identification of these genes is critical to understand how cell sense that peroxisomes need to be degraded, how the microautophagy process is initiated and how peroxisomes are targeted for vacuolar degradation.

Gsa1p has been identified as Pfk1p. It is needed for the initiation of microautophagy. In *gsa1-1*, a step in an initiation step is defective as shown in Fig. 7-4 and this is the only mutant that in this signaling mutant group. Further test of our hypothesis to probe how PFK1 acts in microautophagy process as well as possible participants is crucial for the understanding of microautophagy process.

Gsa7 have been identified in a homotypic vacuolar membrane fusion mutant group. *GSA7* gene has been recovered and it encode a novel protein with no known function in yeast and other species. We propose that it is required for a step at or upstream of homotypic fusion event of vacuolar membrane. We hypothesized that functional role of Gsa7p is to regulate the vacuolar membrane fusion event in microautophagy. The hypothesis I proposed in the first half of this chapter and the possible research experiments will give us an answer for that.

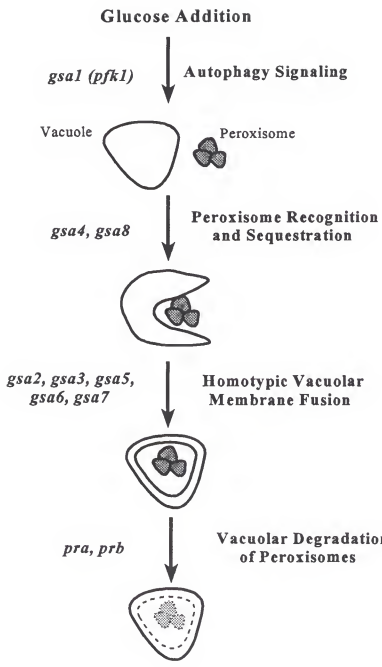


Figure 7-4 Pathway of glucose induced peroxisome microautophagy and it relationship with *gsa* mutants. The microautophagy pathway of peroxisomes during glucose adaptation is depicted here. Gsa1p (Pfk1p) assists signaling the onset of microautophagy while Gsa7p acts at a fusion step of the yeast vacuolar membrane. Other mutants that act in different steps of microautophagy are also included in the figure.

Our data of *gsa* mutants characterization (also see chapter 3) suggest that this genetic model will yield the identities of more unique *GSA* genes. Furthermore, there exists more than eight complementation groups of *gsa* mutants. Further screen and biochemical studies of *gsa* mutants are needed to cover all the regulatory steps and molecules in the microautophagy process and thus more details can be added to the diagram presented in Fig. 7-4. The molecular characterization of microautophagy mutants in *P. pastoris* I have finished has clearly shown that this autophagy study model will aid us to know better about the poorly understood autophagy mechanism. The studies I have done and those of others should make it obvious that this is one of the most dynamic and fascinating study area in the eukaryotic cells.

REFERENCES

Ahlberg, J., and H. Glaumann. 1985. Uptake--microautophagy--and degradation of exogenous proteins by isolated rat liver lysosomes. Effects of pH, ATP, and inhibitors of proteolysis. *Exp Mol Pathol* 42: 78-88.

Angelov, D. N., W. F. Neiss, A. Gunkel, O. Guntinas Lichius, and E. Stennert. 1994. Axotomy induces intranuclear immunolocalization of neuron-specific enolase in facial and hypoglossal neurons of the rat. *J Neurocytol* 23: 218-33.

Arnold, H., and D. Pette. 1968. Binding of glycolytic enzymes to structure proteins of the muscle. *Eur J Biochem* 6: 163-71.

Arvanitidis, A., and J. J. Heinisch. 1994. Studies on the function of yeast phosphofructokinase subunits by in vitro mutagenesis. *J Biol Chem* 269: 8911-8.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1988). Current protocols in molecular biology. K. Janssen, ed. (New York: John Wiley & Sons, Inc).

Baba, M., K. takeshige, H. Baba, and Y. Ohsumi. 1994. Ultrastructural analysis of the autophagic process in yeast: Detection of autophagosomes and their characterization. *J Cell Biol* 124: 903-913.

Berger, S. A., and P. R. Evans. 1992. Site-directed mutagenesis identifies catalytic residues in the active site of Escherichia coli phosphofructokinase. *Biochemistry* 31: 9237-42.

Blangy, D., H. Buc, and J. Monod. 1968. Kinetics of the allosteric interactions of phosphofructokinase from Escherichia coli. *J Mol Biol* 31: 13-35.

Boles, E., J. Heinisch, and F. K. Zimmermann. 1993. Different signals control the activation of glycolysis in the yeast Saccharomyces cerevisiae. *Yeast* 9: 761-70.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-54.

Breitenbach-Schmitt, I., J. Heinisch, H. D. Schmitt, and F. K. Zimmermann. 1984a. Yeast mutants without phosphofructokinase activity can still perform glycolysis and alcoholic fermentation. *Mol Gen Genet* : 530-535.

Breitenbach-Schmitt, L., H. D. Schmitt, J. Heinisch, and F. K. Zimmermann. 1984b. Genetic and physiological evidence for the existence of a second glycolytic pathway in yeast parallel to the phosphofructokinase-aldolase reaction sequence. *Mol Gen Genet* : 536-540.

Chen Zion, M., G. Lilling, and R. Beitner. 1993. The dual effects of Ca2+ on binding of the glycolytic enzymes, phosphofructokinase and aldolase, to muscle cytoskeleton. *Biochem Med Metab Biol* 49: 173-81.

Chiang, H. L., and R. Schekman. 1991. Regulated import and degradation of a cytosolic protein in the yeast vacuole. *Nature* 350: 313-8.

Chiang, H. L., R. Schekman, and S. Hamamoto. 1996. Selective uptake of cytosolic, peroxisomal, and plasma membrane proteins into the yeast lysosome for degradation. *J Biol Chem* 271: 9934-41.

Chiang, H. L., and H. L. Shieh. 1997. Identification of novel vesicles in the cytosol to lysosome targeting and degradation pathway. Page 1781. *37th American Society for Cell Biology Annual Meeting*. Molecular Biology of the Cell, Supplement, Washington, DC.

Clarke, F., P. Stephan, D. Morton, and J. Weidemann. 1985. *Glycolytic enzyme organization via the cytoskeleton and its role in metabolic regulation*. CRC Press, Boca Raton.

Crane, D. I., J. E. Kalish, and S. J. Gould. 1994. The *Pichia pastoris* PAS4 gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. *J Biol Chem* 269: 21835-44.

Cregg, J. M., K. J. Barringer, A. Y. Hessler, and K. R. Madden. 1985. *Pichia pastoris* as a host system for transformations. *Mol Cell Biol* 5: 3376-85.

Cregg, J. M., I. J. van der Klei, G. J. Sulter, M. Veenhuis, and H. Harder. 1990. Peroxisome-deficient mutants of Hansenula polymorpha. *Yeast* 6: 87-97.

Cregg, J. M., T. S. Vedvick, and W. C. Raschke. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris. Biotechnology NY* 11: 905-10.

de Duve, C. 1965. Functions of microbodies (peroxisomes). *J Cell Biol.* 27: 25A-26A.

Dodt, G., N. Braverman, D. Valle, and S. J. Gould. 1996. From expressed sequence tags to peroxisome biogenesis disorder genes. *Ann NY Acad Sci* 804: 516-23.

Dodt, G., and S. J. Gould. 1996. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J Cell Biol* 135: 1763-74.

Dunn, W. A., Jr. 1990a. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J Cell Biol* 110: 1923-33.

Dunn, W. A., Jr. 1990b. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J Cell Biol* 110: 1935-45.

Dunn, W. A. J. 1994. Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends in Cell Biology* 4: 139-143.

Dunn, W. A., Jr., M. K. Raizada, E. S. Vogt, and E. A. Brown. 1994. Growth factor-induced neurite growth in primary neuronal cultures of dogs with neuronal ceroid lipofuscinosis. *Int J Dev Neurosci* 12: 185-96.

Dyson, R. D., J. M. Cardenas, and R. J. Barsotti. 1975. The reversibility of skeletal muscle pyruvate kinase and an assessment of its capacity to support glyconeogenesis. *J Biol Chem* 250:3316-21.

Egner, R., M. Thumm, M. Straub, A. Simeon, H. J. Schuller, and D. H. Wolf. 1993. Tracing intracellular proteolytic pathways. Proteolysis of fatty acid synthase and other cytoplasmic proteins in the yeast Saccharomyces cerevisiae. *J Biol Chem* 268: 27269-76.

Gleeson, M. A., and P. E. Sudbery. 1988. The methylotrophic yeasts. *Yeast* 3: 1-15.

Goldfischer, S. 1996. Peroxisomal diseases: a microscopist looks through the retrospectroscope. *Ann NY Acad Sci* 804: 424-6.

Harding, T. M., A. Hefner Gravink, M. Thumm, and D. J. Klionsky. 1996. Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway. *J Biol Chem* 271: 17621-4.

Harding, T. M., K. A. Morano, S. V. Scott, and D. J. Klionsky. 1995. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. *J Cell Biol* 131: 591-602.

Hauser, K., R. Kissmehl, J. Linder, J. E. Schultz, F. Lottspeich, and H. Plattner. 1997. Identification of isoforms of the exocytosis-sensitive phosphoprotein PP63/parafusin in Paramecium tetraurelia and demonstration of phosphoglucomutase activity. *Biochem J* 323: 289-96.

Heikoop, J. C., M. van den Berg, A. Strijland, P. J. Weijers, W. W. Just, A. J. Meijer, and J. M. Tager. 1992. Turnover of peroxisomal vesicles by autophagic proteolysis in cultured fibroblasts from Zellweger patients. *Eur J Cell Biol* 57: 165-71.

Heinisch, J., and F. K. Zimmermann. 1985. Is the phosphofructokinase-reaction obligatory for glucose fermentation by Saccharomyces cerevisiae? *Yeast* 1: 173-5.

Hill, D. J., A. C. Hann, and D. Lloyd. 1985. Degradative inactivation of the peroxisomal enzyme, alcohol oxidase, during adaptation of methanol-grown Candida boidinii to ethanol. *Biochem J* 232: 743-50.

Hoffman, M., and H. L. Chiang. 1996. Isolation of degradation-deficient mutants defective in the targeting of fructose-1,6-bisphosphatase into the vacuole for degradation in Saccharomyces cerevisiae. *Genetics* 143: 1555-66.

Huang, P. H., and H. L. Chiang. 1997. Identification of novel vesicles in the cytosol to vacuole protein degradation pathway. *J Cell Biol* 136: 803-10.

Ishitani, R., K. Sunaga, A. Hirano, P. Saunders, N. Katsube, and D. M. Chuang. 1996. Evidence that glyceraldehyde-3-phosphate dehydrogenase is involved in age-induced apoptosis in mature cerebellar neurons in culture. *J Neurochem* 66: 928-35.

Jacoby, J., C. P. Hollenberg, and J. J. Heinisch. 1993. Transaldolase mutants in the yeast Kluyveromyces lactis provide evidence that glucose can be metabolized through the pentose phosphate pathway. *Mol Microbiol* 10: 867-76.

Kaiser, C., S. Michaelis, and A. Mitchell. 1994. *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Plainville.

Kalish, J. E., G. A. Keller, J. C. Morrell, S. J. Mihalik, B. Smith, J. M. Cregg, and S. J. Gould. 1996. Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. *EMBO J* 15: 3275-85.

Kato, N. 1990. Formate dehydrogenase from methylotrophic yeasts. *Methods Enzymol* 188: 459-62.

Klionsky, D. J. 1997. Protein transport from the cytoplasm into the vacuole. *J Membr Biol* 157: 105-15.

Kopitz, J., G. O. Kisen, P. B. Gordon, P. Bohley, and P. O. Seglen. 1990. Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. *J Cell Biol* 111: 941-53.

Lardeux, B. R., and G. E. Mortimore. 1987. Amino acid and hormonal control of macromolecular turnover in perfused rat liver. Evidence for selective autophagy. *J Biol Chem* 262: 14514-9.

Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Ann Rev Cell Biol* 1: 480-530.

Liu, H., X. Tan, K. A. Russell, M. Veenhuis, and J. M. Cregg. 1995. PER3, a gene required for peroxisome biogenesis in *Pichia pastoris*, encodes a peroxisomal membrane protein involved in protein import. *J Biol Chem* 270: 10940-51.

Liu, H., X. Tan, M. Veenhuis, D. McCollum, and J. M. Cregg. 1992. An efficient screen for peroxisome-deficient mutants of *Pichia pastoris*. *J Bacteriol* 174: 4943-51.

Lobo, Z., and P. K. Maitra. 1983. Phosphofructokinase mutants of yeast. Biochemistry and genetics. *J Biol Chem* 258: 1444-9.

Luiken, J. J., M. van den Berg, J. C. Heikoop, and A. J. Meijer. 1992. Autophagic degradation of peroxisomes in isolated rat hepatocytes. *FEBS Lett* 304: 93-7.

Matsuura, A., M. Tsukada, Y. Wada, and Y. Ohsumi. 1997. Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae. *Gene* 192:245-50.

McCollum, D., E. Monosov, and S. Subramani. 1993. The pas8 mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells--the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. *J Cell Biol* 121: 761-74.

Mizrachi, Y. 1989. Neurotrophic activity of monomeric glucophosphoisomerase was blocked by human immunodeficiency virus (HIV-1) and peptides from HIV-1 envelope glycoprotein. *J Neurosci Res* 23: 217-24.

Mortimore, G. E., A. R. Poso, and B. R. Lardeux. 1989. Mechanism and regulation of protein degradation in liver. *Diabetes Metab Rev* 5: 49-70.

Moser, H. W., and A. B. Moser. 1996. Peroxisomal disorders: Overview. *Annu Rev Cell Biol* 9: 427-441.

Noda, T., and Y. Ohsumi. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* 273:3963-6.

Nuttley, W. M., R. K. Szilard, J. J. Smith, M. Veenhuis, and R. A. Rachubinski. 1995. The PAH2 gene is required for peroxisome assembly in the methylotrophic yeast Hansenula polymorpha and encodes a member of the tetratricopeptide repeat family of proteins. *Gene* 160: 33-9.

Olson, T. S., S. R. Terlecky, and J. F. Dice. 1992. Pathways of intracellular protein degradation in eukaryotic cells. Pages 89-114 in T. J. Ahern and M. C. Manning, eds. *Stability of protein pharmaceuticals. Part 2: In vivo pathways of degradation and strategies for protein stabilization.* Plenum Press, New York.

Robbins, A. R., R. D. Ward, and C. Oliver. 1995. A mutation in glyceraldehyde 3-phosphate dehydrogenase alters endocytosis in CHO cells. *J Cell Biol* 130: 1093-104.

Roberts, S. J., and G. N. Somero. 1989. Properties of the interaction between phosphofructokinase and actin. *Arch Biochem Biophys* 269: 284-94.

Ronai, Z. 1993. Glycolytic enzymes as DNA binding proteins. *Int J Biochem* 25: 1073-6.

Sakai, Y., A. Koller, L. K. Rangell, G. A. Keller, and S. Subramani. 1998. Peroxisome Degradation by Microautophagy in *Pichia pastoris*: Identification of Specific Steps and Morphological Intermediates. *J Cell Biol* 141: 625-636.

Scott, S. V., A. Hefner Gravink, K. A. Morano, T. Noda, Y. Ohsumi, and D. J. Klionsky. 1996. Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole. *Proc Natl Acad Sci USA* 93: 12304-8.

Shimozawa, N., T. Tsukamoto, Y. Suzuki, T. Orii, Y. Shirayoshi, T. Mori, and Y. Fujiki. 1992. A human gene responsible for Zellweger syndrome that affects peroxisome assembly. *Science* 255: 1132-4.

Shirahama, K., T. Noda, and Y. Ohsumi. 1997. Mutational analysis of Csc1/Vps4p: involvement of endosome in regulation of autophagy in yeast. *Cell Struct Funct* 22:501-9.

Sibirny, A. A., V. I. Titorenko, and B. D. Efremov. 1987. Multiplicity of mechanisms of carbon catabolite repression involved in the synthesis of alcohol oxidase in the methylotrophic yeast *Pichia pastoris*. *Yeast* 3: 233-241.

Smalheiser, N. R. 1996. Proteins in unexpected locations. *Mol Biol Cell* 7: 1003-14.

Sreekrishna, K., R. G. Brankamp, K. E. Kropp, D. T. Blankenship, J. T. Tsay, P. L. Smith, J. D. Wierschke, A. Subramani, and L. A. Birkenberger. 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* 190: 55-62.

Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu Rev Cell Biol* 9: 445-478.

Subramani, S. 1996. Convergence of model systems for peroxisome biogenesis. *Curr Opin Cell Biol* 8: 513-8.

Takei, N., J. Kondo, K. Nagaike, K. Ohsawa, K. Kato, and S. Kohsaka. 1991. Neuronal survival factor from bovine brain is identical to neuron-specific enolase. *J Neurochem* 57: 1178-84.

Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J Cell Biol* 119: 301-11.

Terlecky, S. R., W. M. Nuttley, D. McCollum, E. Sock, and S. Subramani. 1995. The *Pichia pastoris* peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. *EMBO J* 14: 3627-34.

Terlecky, S. R., E. A. C. Wiemer, W. M. Nuttley, P. A. Walton, and S. Subramani. 1996. Signals, receptors, and cytosolic factors involved in peroxisomal protein import. *Ann NY Acad Sci* 804: 11-20.

Thumm, M., R. Egner, B. Koch, M. Schlumpberger, M. Straub, M. Veenhuis, and D. H. Wolf. 1994. Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. *FEBS Lett* 349: 275-80.

Titorenko, V. I., I. Keizer, W. Harder, and M. Veenhuis. 1995. Isolation and characterization of mutants impaired in the selective degradation of peroxisomes in the yeast Hansenula polymorpha. *J Bacteriol* 177: 357-63.

Tolbert, N. E. 1981. Metabolic pathways in peroxisomes and glyoxysomes. *Ann Rev Biochem* 50: 133-157.

Tomlison, E. 1992. Direct colony assay for alcohol oxidase. *Biotechniques* 13: 710.

Treptau, T., R. Kissmehl, J. D. Wissmann, and H. Plattner. 1995. A 63 kDa phosphoprotein undergoing rapid dephosphorylation during exocytosis in Paramecium cells shares biochemical characteristics with phosphoglucomutase. *Biochem J* 309: 557-67.

Tsukada, M., and Y. Ohsumi. 1993. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett* 333: 169-74.

Tuttle, D. L., and W. A. Dunn, Jr. 1995. Divergent modes of autophagy in the methylotrophic yeast *Pichia pastoris*. *J Cell Sci* 108: 25-35.

Tuttle, D. L., A. S. Lewin, and W. A. Dunn, Jr. 1993. Selective autophagy of peroxisomes in methylotrophic yeasts. *Eur J Cell Biol* 60: 283-90.

van der Klei, I. J., W. Harder, and M. Veenhuis. 1991a. Biosynthesis and assembly of alcohol oxidase, a peroxisomal matrix protein in methylotrophic yeasts: a review. *Yeast* 7: 195-209.

van der Klei, I. J., W. Harder, and M. Veenhuis. 1991b. Methanol metabolism in a peroxisome-deficient mutant of Hansenula polymorpha: A physiological study. *Arch Microbiol* 156: 15-23.

van der Klei, I. J., and M. Veenhuis. 1996. Peroxisome biogenesis in the yeast Hansenula polymorpha: a structural and functional analysis. *Ann NY Acad Sci* 804: 47-59.

Veenhuis, M., A. Douma, W. Harder, and M. Osumi. 1983. Degradation and turnover of peroxisomes in the yeast Hansenula polymorpha induced by selective inactivation of peroxisomal enzymes. *Arch Microbiol* 134: 193-203.

Veenhuis, M., W. Harder, J. P. van Dijken, and F. Mayer. 1981. Substructure of crystalline peroxisomes in methanol-grown Hansenula polymorpha: evidence for an in vivo crystal of alcohol oxidase. *Mol Cell Biol* 1: 949-57.

Veenhuis, M., J. P. van Dijken, S. A. Pilon, and W. Harder. 1978. Development of crystalline peroxisomes in methanol-grown cells of the yeast Hansenula polymorpha and its relation to environmental conditions. *Arch Microbiol* 117: 153-63.

Waterham, H. R., Y. de Vries, K. A. Russel, W. Xie, M. Veenhuis, and J. M. Cregg. 1996a. The *Pichia pastoris* PER6 gene product is a peroxisomal integral membrane protein essential for peroxisome biogenesis and has sequence similarity to the Zellweger syndrome protein PAF-1. *Mol Cell Biol* 16: 2527-36.

Waterham, H. R., Y. De Vries, K. A. Russell, W. Xie, M. Veenhuis, and J. M. Cregg. 1996b. The *Pichia pastoris* PER6 gene encodes the yeast homologue of human PAF-1. *Ann NY Acad Sci* 804: 773-4.

Waterham, H. R., M. E. Digan, P. J. Koutz, S. V. Lair, and J. M. Cregg. 1997. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* 186: 37-44.

Wills, C. 1990. Regulation of sugar and ethanol metabolism in Saccharomyces cerevisiae. *Crit Rev Biochem Mol Biol* 25: 245-80.

Wills, C. 1996. Some puzzles about carbon catabolite repression in yeast. *Res Microbiol* 147: 566-72.

Yahraus, T., N. Braverman, G. Dodt, J. E. Kalish, J. C. Morrell, H. W. Moser, D. Valle, and S. J. Gould. 1996. The peroxisome biogenesis disorder group 4 gene, PXAAA1, encodes a cytoplasmic ATPase required for stability of the PTS1 receptor. *EMBO J* 15: 2914-23.

BIOGRAPHICAL SKETCH

I was born in Shanghai, P. R. China in 1964. My father, Gangjun Yuan, is a head master of an elementary school and my mother, Zhengjuan Xu, is a pediatrician. I graduated from the distinguished high school, Shanghai Middle School, in 1981 and was enrolled in the Second Military Medical University majoring medicine. I graduated in 1986 after four-years of study and one year's internship. I did a three-year residency at a hospital in Shannxi, China, and got back to Shanghai in 1989 as an assistant editor of *the Journal of Reproduction and Contraception,* at Shanghai Institute of Planned Parenthood Research. In August 1993, I entered the Department of Anatomy and Cell Biology in the College of Medicine at the University of Florida for a Ph.D. majoring cell and developmental biology. My research focuses on the regulatory mechanism of protein and cell organelle microautophagy in yeast *P. pastoris*.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William A. Dunn, Jr., Chair
Associate Professor of Anatomy
and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John P. Aris
Assistant Professor of Anatomy
and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gudrun S. Bennett
Research Professor of Anatomy
and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Alfred S. Lewin
Professor of Molecular Genetics
and Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1998

Dean, College of Medicine

Dean, Graduate School